EFFICACY AND MODE OF ACTION OF IMMUNE RESPONSE MODIFYING COMPOUNDS AGAINST ALPHAVIRUSES AND FLAVIVIRUSES

Annual and Final Report

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December 1988

Support by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6117

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Department of Microbiology and Immunology
3300 Henry Avenue
Philadelphia, PA 19129

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Medical College of Pennsylvania; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Philadelphia, Pennsylvania 19129	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-C-6117
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62770A	3M1-62770A871	AG	370

11. TITLE (Include Security Classification)
(U) Efficacy and Mode of Action of Immune Response Modifying Compounds Against Alphaviruses and Flaviviruses

12. PERSONAL AUTHOR(S)
Page S. Morahan, Margo Brinton, and Angelo J. Pinto

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final	FROM 1/1/86 TO 12/31/88	1988 December	71

16. SUPPLEMENTARY NOTATION
Annual covers January 1, 1988 through December 31, 1988.

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	RA 1; Immune Response Regulators; Alphaviruses; Flaviviruses
06	15		
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION: Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): 301-663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete. SECURITY CLASSIFICATION OF THIS PAGE

19. Abstract -continued

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Banzi Virus

SUMMARY OF THIRD CONTRACT YEAR

The work of the current year continues to demonstrate broad spectrum antiviral activity of immunomodulators against herpes simplex virus (HSV-2), and Banzi flavivirus, Semliki forest alphatogavirus (SFV), and Caraparu bunyavirus. There were, however, distinct patterns in the sensitivity of the virus infections to immunomodulation. HSV-2 infection was the most sensitive; therapeutic treatment with Ampligen or any of the three types of interferon (IFN) was effective even when the start of treatment was delayed until 48 hr after infection. Against the Banzi virus model, Ampligen, poly ICLC and the three IFNs were active in repeated therapeutic treatment schedules. These compounds were also effective against SFV in repeated early therapeutic treatment schedules. In contrast with their effectiveness against the other viruses, even with repeated prophylactic/therapeutic treatment schedules, the three IFNs and Ampligen were only partially effective against Caraparu virus while poly ICLC gave excellent protection. Combination experiments revealed synergistic antiviral activity against Caraparu virus between the nucleoside analogue ribavirin and gamma IFN. Other combination treatments with Ampligen + poly ICLC, ribavirin + poly ICLC, and poly ICLC + beta IFN produced no synergistic activity against Caraparu virus infection. The difference in sensitivity of Caraparu virus to immunomodulator treatment may relate to our finding that Caraparu virus causes death through massive liver destruction while HSV-2, Banzi virus, and SFV are reported to cause death by central nervous system tissue destruction. To assess whether IFN induction is the common mechanism of action of the various immunomodulators, the effect of anti-IFN alpha/beta antibody treatment on immunomodulator antiviral activity was examined. The results indicated that IFN may be the major component in the antiviral activity of Ampligen and ABMP, and to a lesser extent, CL246,738 and MVE-2. Anti-IFN antibody treatment, however, although effectively neutralizing poly ICLC-induced IFN, did not reduce the antiviral activity of poly ICLC. These data suggest that mechanisms in addition to IFN may be involved in the antiviral efficacy of this compound.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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INTRODUCTION

This research is directed at two of the areas of interest for developmental research on immune modifying compounds against viruses of military importance.

1. Evaluate compounds for modulation of specific and nonspecific immune responses in normal B6C3F1 female mice. We are using drug treatment regimens that have been shown to be effective in the in vivo antiviral tests by us and others. We have focused on in vitro/ex vivo tests for intrinsic and extrinsic macrophage antiviral activity, macrophage activation, and natural killer cell activity.

2. Establish the efficacy of prophylactic and/or therapeutic treatment with immunomodulators alone and in combination with antiviral drugs against alphavirus, flavivirus, bunyavirus and herpesvirus infections in vivo. We are evaluating and characterizing the antiviral efficacy in normal mice, and we are developing systems to evaluate a few regimens of particular interest in mice that have been selectively depleted of various nonspecific effector cell populations.

BACKGROUND

Infections caused by viruses belonging to the Alphatogaviridae, Arenaviridae, Bunyaviridae, and Flaviviridae families are an important health problem worldwide, where they frequently cause significant human morbidity (21). Complete control will undoubtedly require a multifaceted approach, including better insect and rodent vector control, improved sanitation, effective vaccines, and development of safe and effective prophylactic/therapeutic treatment with antivirals, which inhibit virus multiplication, and immunomodulators, which modify host resistance. Development of antiviral treatment of RNA viruses has lagged behind drug development for DNA herpesviruses, where a number of clinically licensed efficacious immunomodulators and nucleoside analogs have been described (14,15). Ribavirin has been shown to be an effective antiviral against a few RNA viruses in animal models and is licensed for a few clinical settings (8,24,31,32,33 42). Considerable research in recent years has also documented that many RNA viruses are inhibited by a variety of immunomodulators, many of which are interferon (IFN) or IFN inducers (13,23,36,39,41,74,75,80). Importantly, combinations of two different nucleosides or immunomodulators have clearly been shown to produce synergistic effects (28,29,31). Most of these studies have been performed only in vitro (31,42,53). There is evidence, however, of synergy in vivo for ribavirin combined with the IFN inducer poly ICLC against Rift Valley fever virus (RVF) infection in mice (38), and for ribavirin and specific antibody against Lassa fever virus in monkeys (34). Antiviral activity in mice can also be enhanced if nucleosides such as ribavirin or immunomodulators such as muramyl dipeptide (MDP) are delivered in liposomes (8,24,36,37,40,).

Neither the predominant mechanisms of action of these various compounds (or their combinations), nor the optimum treatment regimens (dose, schedule, route) have yet been established. It has been well documented that immunomodulators can exert "yin-yang" effects on host resistance varying with the compound injection dose, route and schedule in relation to virus or tumor

challenge (30,67,77,83). Nucleosides have toxic properties (9,10) and can also alter the lymphoreticular/hematopoietic system and thus alter the toxicity or the protective action of immunomodulators (14). Thus, systematic evaluation of antiviral efficacy and immunomodulatory activity of promising compounds and compound combinations is necessary before the best clinical treatment can be developed. Additionally, the mode of action of the effective compounds has had little attention.

For these reasons, the current study has evaluated, in mouse model systems, different regimens of immunomodulator treatment on antiviral efficacy against Alphatoga-, Bunya-, and Flaviviridae and has attempted to correlate antiviral protection with antiviral effector mechanisms. Specifically, we have focused on drug-induced changes in macrophage (Mϕ) activation, as assessed by antitumor activity and ectoenzyme phenotype, and natural killer cell (NK) activity. We have also used several selective depletion systems in order to determine the contribution of NK cells, tissue Mϕ, circulating monocytes, and IFN to the antiviral action of immunomodulators. These are mechanisms which have been well documented to be important in resistance to virus infections in experimental animals (2,3,59,60,63,84,91,93). Our results demonstrate that several immunomodulators are effective broad spectrum antiviral agents in vivo. Thus, a variety of immunomodulators may be useful in prophylactic or early therapeutic treatment of several severe viral infections.

RATIONALE

The rationale for this research is that: (i) better methods are needed for treatment of RNA virus infections; (ii) immunomodulation provides a potent new modality of antiviral prophylaxis and therapy; (iii) but, immunomodulation can also produce adverse effects. Thus, there is a need for systematic evaluation of immunomodulators against diverse viral infections, together with more precise delineation of their mechanisms of action on nonspecific immune effector cells and immunoregulatory networks under carefully controlled standard systems.

A variety of potentially useful immunomodulators are being characterized for immunomodulatory activity in the B6C3F1 female mouse animal model that is widely accepted for immunotoxicological and immunopharmacological preclinical evaluations. A battery of standardized nonspecific immune function assays on Mϕ and NK cell activation are being performed. Novel immunomodulators are being compared directly with "classic" immunomodulators in regard to these nonspecific immune functions and antiviral efficacy against a variety of RNA virus infections. For comparison, antiviral efficacy against HSV-2 infection is used as a "gold standard", because the mechanisms of natural host resistance and protective immunomodulatory treatment against HSV have been extensively characterized. A spectrum of systemic RNA virus infections has been selected for study, in order to establish if broad spectrum protection can be produced against viruses that possess different replication and pathogenesis patterns. These include Semliki forest strain L10 alphatogavirus, Banzi flavivirus, and Caraparu bunyavirus; all of which produce significant mortality in adult mice after i.p. infection, but can be worked with under Class II biohazard conditions.

EXPERIMENTAL METHODS

Mice. Virus free, barrier raised, 6 week old female B6C3F1 mice were purchased from Taconic Farms, shipped in filter crates and housed in autoclaved micro-isolator cages (MCP) or PLAS - LAB isolator chambers (Wistar). Usually, two mice from each shipment were bled on arrival, two more at one week, and periodically thereafter for testing to ensure no intercurrent viral infections had occurred. Mouse sera were tested for sero-conversion to MHV and Sendai viruses by the ELISA test (Biocon Labs, Rockville, MD). No sero-conversion for these viruses has been observed in the last 36 months.

Immunomodulators: Table 1 lists the immunomodulators used in this study. C. parvum (Burroughs Wellcome Co., Research Triangle Park, NC) was injected into mice i.p. at 35 mg/kg 7 days prior to cell harvest or infection of mice. MVE-2 (Hercules, Inc., Wilmington, DE) was dissolved in phosphate-buffered saline to a final inoculation concentration of 50 mg/kg and was administered i.p. to mice 1 day prior to virus infection. rMuIFN-G (courtesy of Genentech, South San Francisco, CA), rHuIFN-A A/D (courtesy of Hoffman La Roche, Nutley, NJ), rMuIFN-B (courtesy of Toray Industries, Tokyo, Japan), rMuCSF-GM (courtesy of Behringwerke, Frankfurt, West Germany) and rHuCSF-M (courtesy of Dr. Peter Ralph, Cetus Corporation, CA) were all injected i.p. in a vehicle consisting of phosphate-buffered saline containing 0.2% bovine serum albumin (BSA). Each time an IFN was prepared for injection, it was titered simultaneously for antiviral activity and titers calculated based on the titers of standard reference IFN used in each assay. Ampligen (provided to USAMRIID by Dr. Paul Tso, Johns Hopkins, Baltimore, MD) was dissolved in physiological saline, heated at 67°C for 16 hours and then at 37°C for 1 hour before being injected i.p. Another preparation of Ampligen (courtesy of HEM Research) was supplied as a lyophilized material and was prepared by reconstitution in distilled water. Poly ICLC (provided to USAMRIID by Dr. Hilton Levy, National Cancer Institute) was diluted to the desired concentration in physiological saline. CL246,738 (courtesy of Lederle, Pearl River, NY) was prepared in distilled water and administered per os. The three pyrimidinones, ABPP, AIPP and ABMP were received courtesy of Dr. Harold Renis, Upjohn Co., Kalamazoo, MI. All were suspended in 1% carboxy-methylcellulose (CMC). To ensure a uniform suspension, pyrimidinone preparations were vortexed vigorously just prior to i.p. or per os inoculation. GE-132 (USAMRIID) was ground and suspended in 1% CMC (for per os. administration) or Tween 80 (for i.p. administration). Ribavirin and its analog were dissolved in PBS and injected i.p. GLA-60 (courtesy of Dr. Chiaki Nishimura, Kitasato University, Tokyo, Japan) was injected i.v. after being prepared in PBS.

Viruses. Each virus pool was prepared somewhat differently, in order to maximize the titer of virus produced. The in vitro and in vivo titers of the various viruses are presented in Table 2. Herpes simplex virus type 1 (HSV-1 Kos) and type 2 (HSV-2 MS) were prepared in either secondary rabbit kidney fibroblasts or Vero cells, by infecting cells with a low multiplicity of infection (m.o.i.) , and harvesting the cultures when more than 75% of the cells showed cytopathic effect (CPE) (61). The pools consisted of cell-associated virus that was clarified of cellular debris by low speed centrifugation. Both HSV-1 and HSV-2 were titered on Vero cells with 2% methylcellulose overlay, and the titers were about 2×10^8 plaque forming units (PFU/ml) for HSV-1 and 8×10^6 PFU/ml for HSV-2.

Pools of alphaviruses, flaviviruses, and bunyaviruses were made in newborn CD-1 mice. In all cases two to four day old mice were inoculated with 0.02 ml of virus by the intracerebral route. When moribund, the mice were sacrificed by decapitation and their brains or livers removed and frozen on dry ice. Subsequently, clarified 10% (wt/vol) tissue homogenates were made, aliquoted, and stored frozen at - 70°C.

A stock of Pichinde virus (strain Co An 3739) propagated in Vero cells was obtained from Dr. Pat Repik. A pool was prepared by infecting Vero cells with an m.o.i. of 0.0001, harvesting cell associated virus after three days of incubation by distilled water lysis and one freeze thawing to disrupt cells, followed by clarification of the fluid by low speed centrifugation and storage at -70°C. A suckling mouse brain pool (SMB pool 1, October 1977) of Semliki forest virus (SFV) L10 strain was received from USAMRIID, diluted 10-fold and frozen at - 70°C. It had a titer of 6.8×10^7 PFU/ml on BHK21 cells, and an i.p. LD_{50} of $10^{7.2}$, or about 1 PFU/$LD^{5}0$ in adult B6C3F1 mice. A seed stock of the flavivirus, Banzi virus, strain SA M336, mouse passage 9, was obtained from Dr. Robert Shope, Yale University. Mice were inoculated with a 1:2 dilution of this stock and were sacrificed 3 days after infection. The brain pool prepared contained an infectivity titer of 2.0×10^8 PFU/ml as assayed by plaque titration on BHK 15 cells.

A seed stock of a second mouse passage of the bunyavirus, Caraparu, strain Be AN 3999, was obtained from Dr. Shope, Mice were inoculated intracerebrally with a 1:100 dilution of this stock and sacrificed 48 hr after infection. The livers were removed and used to prepare a clarified 10% (wt/vol) liver homogenate. No plaques were obtained with this virus on BHK-21, BHK-15 or MK2 cell monolayers. The pool had an LD_{50} of $10^{2.4}$ in adult B6C3F1 mice inoculated by the i.p. route. A seed stock of the bunyavirus, Oriboca, strain BeAn 17, mouse passage 12, was obtained from Dr. Shope. Mice were inoculated with a 1:100 dilution of this stock and sacrificed 48 hr after infection. A 10% liver virus pool was prepared and had an infectivity titer of 4×10^6 PFU/ml as assayed on BHK-21 cells.

Peritoneal Mϕ Preparation. Mϕ were harvested by flushing the peritoneal cavity with 5ml of cold Dulbecco's phosphate-buffered saline (DPBS) (Gibco) containing 2 units/ml preservative-free heparin (Invenex Laboratories, Melrose Park,IL), and 50μg/ml gentamicin (Gibco). The peritoneal cells were counted in a Model ZM Coulter Counter (Hialeah, FL), centrifuged at 250 x g for 10 minutes at 5°C and resuspended to a concentration of 2.5×10^6 cells/ml in complete EMEM containing 50μg/ml gentamicin. Slides for cell differentials were prepared in a Cytospin (Shandon-Southern, Sewickley, PA) and were stained with a modified Wright's stain (Geometric Data, Wayne, PA).

Preparation of Kupffer cells (KC). Mice were sacrificed by cervical dislocation, their abdominal skin retracted and their thoracic and peritoneal cavities exposed. A sterile 25-gauge 1 inch needle butterfly infusion set was inserted into the inferior vena cava to the level of the renal artery. To wash the liver free of blood, the liver was perfused with 10 ml Hanks' balanced salt solution (HBSS), Ca^{++}, Mg^{++} free, containing 25 mM HEPES buffer and 1 mM EDTA. To allow for hepatic efflux, the portal vein was cut just prior to perfusion. When this perfusion was properly done the liver was distended and rapidly

blanched to a cream color. Any portion not completely blanched was discarded and the washed liver was then excised and placed in a plastic bag containing HBSS, Ca^{++}, Mg^{++} free, supplemented with 25 mM HEPES buffer, 1 mM Ca^{++} , 0.05% collagenase, 50 U/ml DNAse I and 1.5% BSA. This bag was sealed and placed in a Stomacher lab blender, an instrument which uses alternating paddles to compress the bag and help disperse the liver cells. Use of the Stomacher allowed 10 livers to be processed simultaneously, and the blending action did not reduce cell viability. After blending for 3 minutes, the bag was removed and placed in a 37°C shaker incubator for 45 minutes. The resulting liver digest was filtered through 50-gauge stainless steel mesh and washed 2x with HBSS, ^{++}Ca, ^{++}Mg free 25 mM HEPES at 500 x g at 5°C. Most parenchymal cells were removed by 2 sequential centrifugations at 50 x g for 4 minutes. After a final centrifugation at 500 x g, the liver cells were resuspended in 15 ml of 30% metrizamide in Gey's balanced salt solution. Nine ml of HBSS, Ca^{++}, Mg^{++} free, 25 mM HEPES was then added to give a final concentration of 18% metrizamide. This suspension was overlaid with 5 ml of the HBSS and the tubes were then centrifuged at 1400 x g for 20 minutes at 5°C. Liver nonparenchymal cells (NPC) remained at the metrizamide - HBSS interface while most of the remaining parenchymal cells, contaminating red blood cells and debris pelleted. The NPC fraction was removed, washed 3x in the HBSS and resuspended in complete EMEM. KC were isolated by selective adherence from this NPC fraction, or from the enriched KC fraction obtained from the other procedures detailed in the Results.

Mϕ Ectoenzyme Assays. Peritoneal cells were allowed to adhere in 35mm-diameter well plates at a concentration of 2.5 x 10^6 cells/ml/well for 2 hours at 37°C in a 5% CO_2-air mixture. Afterwards, nonadherent cells were removed by three washes with cold DPBS. The adherent cells were lysed with 0.5% Triton-X-100 in distilled water (200µl/well). The lysates were frozen at -20°C until assayed, using 100ul for alkaline phosphodiesterase I (APD), 20 µl for 5'-nucleotidase (5'N), and 60 µl for protein determination. Protein concentration was determined using the Bio-Rad procedure (Bio-Rad Laboratories, Rockville Center, NY). 5'N specific activity (S.A.) was determined using 0.15 mM ^3H-adenosine monophosphate (AMP) as the substrate and p-nitrophenyl phosphate as the competitive inhibitor of phosphatase activity (1,58). The APD S.A. was assessed as previously described (1,58) using 1.5 nM p-nitrophenyl thymidine-5'-monophosphate as the substrate. The S.A. was determined using the extinction coefficient 12000 for p-nitrophenyl and was expressed as n moles of p-nitrophenyl produced per milligram protein per minute at 37°C.

Intrinsic Mϕ Virus Interactions. Resident (Res) peritoneal Mϕ were obtained by our usual lavage procedures, washed and allowed to adhere for 2 hr. The adherent Res Mϕ were cultured for 24 hours before infection. For infection with HSV, the number of Mϕ present was estimated by counting nuclei obtained after treatment of cells with cetrimide (85). The amount of virus was adjusted to provide the appropriate m.o.i., which was usually about 3 to ensure infection of all the Mϕ. Virus was allowed to adsorb for 1 hr, nonadsorbed virus was removed by washing, and the Mϕ were cultured at 37°C. For some experiments, the amount of non-adsorbed virus was estimated by titrating the amount of infectious virus in the supernatant. The amount of adsorbed but not yet eclipsed virus was estimated by titrating the amount of cell-associated infectious virus present at 1 hr. The Mϕ cultures were observed for cytopathic effect (CPE), and total virus yield (cell plus supernatant fluid) or supernatant virus yields were

obtained and frozen until assayed by plaque titration. HSV samples were sonicated prior to titrating. Infectious virus yields were expressed as PFU per culture, and as PFU per cell in order to correct for differences in cell numbers in the cultures. Various controls were usually included in the Mϕ-virus experiments. A fully permissive cell line (such as Vero cells for HSV-1) was infected simultaneously with Res Mϕ in order to test the viability of the virus preparation. A thermal inactivation control, consisting of the same concentration of virus as used in the Mϕ infection, was incubated and samples were periodically titrated to establish the rate of viral inactivation in the absence of Mϕ.

Extrinsic Mϕ Virus Interactions. This procedure was similar to that previously developed by us with HSV-2 (66). For assays using HSV-1, 2.5×10^5 Vero cells were cultured overnight in 16mm tissue culture wells of 24-well plates. A sample of Vero cells was lysed with cetrimide and the nuclei counted, in order to determine the appropriate number of peritoneal cells to add to provide a ratio of ca. 1-3 Mϕ:Vero cell. The effector peritoneal cells were obtained by lavage from untreated mice (Res Mϕ) or mice treated with various immunomodulators. All immunomodulators were inoculated by a schedule identical to the prophylactic schedule (time, dose, and route) that provided antiviral protection, and the peritoneal cells were obtained on what would have been the day of i.p. challenge with HSV. By this procedure, the extrinsic antiviral activity of Mϕ was assessed at an early time in infection during which Mϕ antiviral activity appears to be important (63).

To perform the assay, Vero cells were infected with 100 PFU of HSV-1, virus was adsorbed for 1 hr, and nonadsorbed virus removed. Three cultures, that received no further treatment, served as controls. The appropriate number of peritoneal cells was added to the other wells containing HSV-infected Vero cells, taking into consideration differences among immunomodulator elicited peritoneal cells in regard to the proportion of cells that are Mϕ and the percent of Mϕ that adhere. After 2 hr, the nonadherent cells were removed by washing twice, and media was added. Samples containing Vero cells alone and Vero + Mϕ were counted, in order to calculate the actual number of effector cells present. Cultures were incubated for 48 hr before freezing cells and supernatant fluid to determine the total yield of HSV. The total PFU/culture was determined by plaque formation on Vero cells. Generally, three cultures per experimental group were used, and differences among the geometric means of the virus yields were analyzed by the paired t-test. Experiments with the other viruses were performed similarly, except that different target cells were used depending upon in which cells the viruses grew optimally.

Antiviral Protection Studies. B6C3F1 mice were usually randomized into experimental groups by using a computer generated random numbers series. Generally, 15 mice were used in the placebo control group, and 10 mice in each experimental group. A LD_{50} titration was performed simultaneously with each experiment, in order to ensure the appropriate number of LD_{50} doses was used for infection. A simultaneous *in vitro* titration of PFU/ml of the pool was also often performed. Mice were treated with the compounds at the doses, routes and schedules indicated on each table of results. Infected mice were monitored daily for signs of clinical illness (ruffled fur, hunched back, paralysis, CNS symptoms) and mortality. Obviously moribund mice were sacrificed, and their day of death designated as the next day. Mice were observed usually for 21 days.

The percent mortality and median survival time in days (MST) of each group were calculated.

In order to minimize data presentation, generally only the lowest doses tested that exhibited significant antiviral activity are reported. Where several experiments had placebo control groups that did not differ markedly in mortality or MST, the data for only the experimental groups are presented, each with the appropriate statistical analysis as compared with the placebo controls in the particular experiment. The range of mortality and MST data for the individual experiments are shown in the footnotes.

Statistical Analysis. Statistical significance for the immunomodulator data was determined with an Apple IIe microcomputer using the Chi Square test included in the Applestat statistical package (87). This test is not overly conservative as is the Fisher's exact test or Chi Square with Yate's correction factor, and thus can point out immunomodulator regimens with partial activity (7). The median survival time (MST) was calculated, and the survival distribution data were analyzed with the life-tables method and the Lee-Desu method of group comparison (49), using the SPSSX package on the VAX. This procedure allows the most appropriate analysis of survival data with censored observations (i.e. mice still alive at the end of the observation period).

RESULTS Of THIRD YEAR OF THE CONTRACT

Immunomodulator Profiles

Activation of peritoneal Mϕ by treatment of mice with immunomodulators. Throughout the third year of study, we continued evaluating new immunostimulatory compounds for general modulation of nonspecific immunity (Mϕ activation). Mice were treated with murine recombinant granulocyte-macrophage colony stimulating factor (rMuCSF-GM) or human recombinant macrophage colony stimulating factor (rHuCSF-M) and the effects on peritoneal cell population numbers and differential, and on Mϕ ectoenzymes were assessed. Treatment of mice with rMuCSF-GM at 50 μg/mouse i.p. resulted in partial Mϕ activation, as evidenced by a significant decrease in APD specific activity although there was no significant change in 5'N specific activity (Table 3).

Single treatment (i.p.) of mice with rHuCSF-M at 4×10^6 U/ml i.p. also resulted in partial Mϕ activation in one experiment, as evidenced by a significant decrease in 5'N specific activity and a marked reduction in APD specific activity (Table 4). This effect was not observed in the repeat experiment. In both experiments with rHuCSF-M, the immunomodulator had no effect on peritoneal cell population numbers or differential cell counts; however, Mϕ appeared to be dividing in the slides stained for the differential counts in the second experiment.

Thus, single i.p. treatment with either rMuCSF-GM or rHuCSF-M does not appear to cause major Mϕ activation, as assessed by ectoenzyme phenotype changes.

Intrinsic antiviral activity of Res and immunomodulator activated Kupffer cells (KC). We have previously shown that Res and immunomodulator activated KC exhibited marked intrinsic antiviral activity against HSV (65). We have now evaluated whether immunomodulator activated Mϕ exhibited enhanced intrinsic antiviral activity to Pichinde virus, or any differences in response to virus infection, as compared with Res KC (Table 5). Both Res and immunomodulator activated KC exhibited marked intrinsic antiviral resistance to Pichinde virus, as compared with permissive Vero cells. There were no significant differences in the degree of antiviral resistance between Res KC or immunomodulator activated KC. Furthermore, none of the KC tested showed significant cytopathic effect after virus infection.

Extrinsic antiviral activity of Res and immunomodulator activated peritoneal Mϕ against Semliki forest (SFV) and Pichinde viruses. We have previously demonstrated the ability of Res and immunomodulator activated peritoneal Mϕ to exhibit extrinsic antiviral activity against HSV replication in Vero cells (65). This year, the ability of various Mϕ to inhibit Pichinde and Semliki forest virus replication in normally permissive cells was evaluated. Res Mϕ showed significant extrinsic antiviral activity against Pichinde virus infection in permissive Vero cells (Table 6). Mϕ populations activated by treatment of mice with immunomodulators, with the exception of the ABMP per os treated group, also showed significant inhibition of virus yield in Vero cells. However, the immunomodulator activated Mϕ did not show a significant enhancement extrinsic antiviral activity. Less extrinsic activity was observed against SFV infection of BHK cells (Table 7). Of the three immunomodulators tested (C. parvum, rMuCSF-GM and ABPP), only peritoneal Mϕ from ABPP treated mice showed significant extrinsic antiviral activity.

With Pichinde and HSV infections, there was a trend for Mϕ obtained from immunomodulator treated mice to show increased extrinsic activity as compared with Res Mϕ. However, greater numbers of animals must be tested before definitive conclusions can be drawn concerning the quantitative relationship between extrinsic antiviral activity of immunomodulator activated Mϕ as compared with Res Mϕ.

Kupffer Cell (KC) Studies

In order to decrease the persistent contamination of KC with liver endothelial cells (LEC) that we have previously reported (65), we have continued to evaluate novel ways to isolate KC free of the other cell types in the nonparenchymal cell (NPC) fraction. The major contaminant in our NPC preparations has been the LEC which have a density similar to KC; therefore, the two cell types copurify on metrizamide density gradients which is one of the two standard procedures for separating NPC from liver parenchymal cells. LEC have presented a problem to us because, like KC and unlike some other endothelial cells, LEC are an adherent cell type even when cultured for short periods of time at 37°C in serumless medium. Thus, after metrizamide gradient centrifugation and adherence, the attached cells at 2 hr are approximately 30% KC and 70% LEC. Our results using metrizamide density gradient centrifugation, followed by adherence have shown an average of 7×10^6 NPC recovered per mouse (range 2.6-1.5 x 10^7) which is consistent with NPC recoveries reported by others

(50,93,94,96). These data show there is a potential yield of 1-5 x 10^6 KC per mouse. The LEC do not appear to survive well in culture. At 24 hr, the viable attached cells are primarily KC (about 60%); however, the LEC cell debris remains attached despite washing. Therefore, there is still the problem of (i) contaminating protein and other cell macromolecules from LEC, and (ii) KC being cocultivated with this debris from LEC, which may alter their functions. Thus, LEC may affect certain parameters (such as ectoenzyme phenotype, and intrinsic and extrinsic antiviral activity) when we are analyzing the activity of the population as a whole rather than activity on an individual cell basis. For this reason, we have proceeded with much more caution than other studies where KC purity is not so essential.

Unlike other Mϕ populations, one cannot report pure mouse KC populations on the basis of: (i) positive stain for nonspecific esterase, (ii) possession of an Fc receptor for IgG, and (iii) nonspecific phagocytosis of particles such as latex beads, because LEC can also stain positively for esterase, possess Fc receptors, and are capable of nonspecific phagocytosis. Consequently, we have spent a great deal of time assessing LEC contamination in our NPC fraction and we have also used more specific markers for both KC (Fc receptor mediated phagocytosis) and LEC (uptake of fluoresceinated ovalbumin). We have recently obtained a monoclonal antibody that is reported to be specific for KC within the liver (46,47); this should also be useful in our characterization of KC and LEC. The various separation procedures, all of which were used in addition to initial density separation on metrizamide, that we have evaluated this year are discussed separately below.

1) Elutriation: We performed a small number (5) of experiments on the use of elutriation to separate KC from LEC. Although we did enrich for KC, the number of initial cells required for efficient elutriation with our Sanderson chamber (at least 10^8), the number of cells lost and the problem of cell clumping, which interfered with efficient separation, made this procedure hard to reproduce. At the time we were doing these studies, we learned of the Brinkman cellsep system, which appeared to be a more feasible method for small numbers of KC, and thus we moved to evaluation of that system.

2) Separation at unit gravity: The Brinkman cellsep apparatus, which, like the elutriator, separates cells on the basis of size, by sedimentation at 1 x g through a linear gradient of bovine serum albumin (BSA). In theory, KC, being two to three times larger than LEC, should sediment much faster and be collected from the bottom of the gradient after a two hour incubation. This procedure initially looked very promising. We were able to get a KC fraction of over 80% purity, as we previously reported (15 October 1988 quarterly report). However, the yield was very low (ca. 0.4 x 10^6 enriched preparation containing 80% KC). Moreover, over the course of 25 experiments, in which we varied a number of parameters (FCS and Ficoll gradient materials, 4°C versus room temperature, cell concentration, etc.) this system was not reproducible. The major source of the variation was cell clumping. Clumps of cells sediment rapidly, disrupting the linear gradient, causing smaller cells to sediment much more quickly than they normally would, resulting in a poor separation. We were unable to solve the problem of cell clumping despite (i) treating the cells with high concentrations of DNAse, (ii) using separation medium which contained EDTA, and (iii) performing the cell separation in the cold.

3) Sepracell MN: Sepracell MN is a colloidal based separation medium which has been effectively used to separate human peripheral blood monocytes from lymphocytes. These two cell types have similar densities and copurify on ficoll-hypaque density gradients, which is analogous to our situation where liver KC and LEC, having similar densities, copurify on metrizamide density gradients. Working with various concentrations of Sepracell MN, however, we were unable to effectively separate mouse KC from LEC.

4) C3b Rosetting: Another way to differentiate KC from LEC is by C3b rosetting. KC, having receptors for the complement component C3b, readily form rosettes with complement coated particulates. We have used this characteristic to effectively separate KC from LEC. KC, when incubated with sheep red blood cells (SRBC) coated with IgM antibody and complement readily attach to the antibody-complement complex by their C3b receptors. The rosetted KC, now surrounded by SRBC, are much heavier than the nonrosetted cells, and can be separated from them by another density gradient centrifugation through metrizamide. Using this procedure, we have attained KC purities of about 70%, with the majority of the contaminating cells being lymphocytes, which are easily separated from KC by adherence. We believe this separation technique is a good one since by morphological standards, 90% of our adherent cells are KC. This is the best purity that we have been able to obtain with any of the procedures we have attempted, and the cells look healthy in culture for at least a week. We are still working to increase the yield, although we are recovering 55% of the total C3b rosetted cells. Thus, this procedure, unlike the cellsep, does allow us to recover about 1.0×10^6 KC/mouse which is a workable number if we modify our assays to a microtiter system. Of importance, we have gotten consistent results in the 5 experiments performed thus far.

Studies on Pathogenesis and Plaque Formation with Caraparu Virus

After i.p. infection with Caraparu, 4-6 week old B6C3F1 mice died between 4 to 6 days after infection. No clinical symptoms were observed until a few hours prior to death, when mice appeared lethargi- and had a lowered body temperature. To obtain a more precise picture of the disease process, plasma virus and enzyme titers as well as tissue histopathology were assessed.

Plasma virus titers. Plasma was collected daily from mice after infection by the i.p. route with one LD_{90} dose of virus. Viremia was first detected on day 2 (3×10^5 LD_{50}/ml) and virus titers increased thereafter (3×10^8/ml on day 4) (Fig. 1). Virus titers were determined in 3 day old suckling mice, because we were not able to develop a plaque assay for Caraparu virus.

A number of different cell lines were used in numerous attempts to find a tissue culture plaque assay system for Caraparu virus. In each instance, the other mouse bunyavirus, Oriboca, was used as a positive control. Plaquing in BHK21, strain 2, BHK15, LLC-MK2 and CER cells yielded similar results. Plaques were observed in each of these cell lines by 3 to 4 days after infection with Oriboca virus, but no plaques were ever observed with Caraparu virus. Since Caraparu virus is liver tropic in the intact animal, a mouse liver cell line, NCTC, clone 1469, was also tested. The NCTC cells detach before forming a confluent monolayer. Therefore, infectivity assays with those cells were carried

out with liquid as well as solid overlays. Tissue-Tak attached NCTC cells were also tried with no success. No Caraparu virus-specific cytopathology was detected under any conditions in any of the cells. However, it seems likely that Caraparu virus may have replicated in most if not all of these cells, since virus growth was observed in BHK21 strain 2 cells as detected by ^{3}H-uridine labeling of virion RNA. Attempts were also made to prepare virus-specific antibody to use in a fluorescent focus forming assay. The virus preparation used for immunization was not pure. Extensive adsorbtions with mouse liver powder were unsuccessful in removing all of the non-viral background fluorescence and no virus-specific fluorescence was observed.

Plasma enzyme levels. The plasma levels of SGPT, SGOT and LDH were measured in uninfected mice and in infected mice on days 1 through 4 after infection with Caraparu virus (Fig. 1). Elevated levels of these three enzymes in plasma indicate liver cell damage. Slight increases in the levels of all three enzymes were observed on days 1 and 2 after infection. By day 3, the plasma levels of all the enzymes were substantially increased, especially LDH. The levels of SGOT and SGPT were dramatically increased on day 4 after infection, indicating the presence of marked liver damage.

Histopathology. Gross observation of the organs of Caraparu virus infected mice sacrificed on days 1 through 4 after infection revealed a transient enlargement of the spleen on day 1 and a pale liver beginning on day 3. Fixed, stained thin sections of liver were examined under a light microscope. All livers appeared normal on days 1 and 2 after infection (Fig. 2). On day 3, all livers showed coagulation necrosis, with approximately one half to three quarters of the liver sections examined showing focal necrosis. This necrosis was characterized by the disruption of hepatocyte nuclei and plasma membranes and the coagulation of cytoplasm. Few, if any, inflammatory cells were observed. By day 4 after infection, massive coagulation necrosis was observed in essentially 100% of the liver sections examined. Again, no inflammatory cells were observed. Brains, examined at the same intervals after infection, appeared completely normal. These findings indicate that the cause of death in animals infected with Caraparu virus is due to massive liver destruction.

Broad Spectrum Antiviral Efficacy of Treatment of Mice with Immunomodulators

During this third contract year, we have continued to evaluate the antiviral efficacy of a variety of immunomodulators against herpesvirus, flavivirus, alphatogavirus, and bunyavirus infections. We have focused on evaluations of therapeutic regimens.

Antiviral efficacy of immunomodulators against HSV-2 infection. We have continued to use the HSV-2 infection model as a standard against which to compare the antiviral activity of the various immunomodulators against RNA viruses. The mismatched polyribonucleotide, Ampligen, was shown to be quite effective upon repeated therapeutic administration beginning as late as 2 days after infection (Table 8). In addition, when the start of repeated therapeutic therapy was delayed to day +2 after infection all three IFNs still exerted excellent antiviral activity. In contrast with our other models, we found no major

differences among rHuIFN-A, rMuIFN-B, or rMuIFN-G against lethal HSV-2 infection.

Antiviral efficacy of immunomodulators against Banzi flavivirus. We have previously indicated that Banzi virus is a very sensitive model infection in which to evaluate immunomodulators (64,65). Repeated therapeutic treatment with either Ampligen or poly ICLC, beginning 24 hr after infection, provided significant antiviral activity against Banzi virus (Table 9). Moreover, if treatment with either compound was delayed until 48 hr after infection, partial protection as evidenced by a significant increase in MST was still observed.

Results of therapeutic treatment of Banzi virus infection with the recombinant IFNs indicated that rHuIFN-A and rMuIFN-B were considerably more effective against Banzi virus than was treatment with rMuIFN-G (Table 9). When rMuIFN-G was given therapeutically on a daily schedule (day 0 to +6 or day +1 to +6), this IFN did not protect mice against Banzi virus. However, the same treatment schedules with rHuIFN-A and rMuIFN-B provided significant antiviral activity. Unlike the results with HSV-2 infection, delaying the start of treatment with rHuIFN-A or rMuIFN-B to day +2 after infection with Banzi virus resulted in a complete loss of antiviral activity.

We also tested two pyrimidinones, ABMP and ABPP, in single prophylactic, single therapeutic, and repeated therapeutic treatment schedules against Banzi virus (Table 9). ABMP was completely ineffective in all three treatment regimens, whether given i.p. or per os. ABPP significantly increased the MST of all treated groups, and also significantly reduced mortality in the group that received repeated i.p. therapy.

In previous studies with rHuCSF-M we found no antiviral activity in a day -1 to day +6 treatment schedule (65). This year we showed that even beginning treatment 4 days before infection did not increase the efficacy of rHuCSF-M (Table 9).

Antiviral efficacy of immunomodulators against Semliki Forest virus infection. Like Banzi virus, infection with SFV has also been a very sensitive model in which to evaluate immunomodulators (65). During this year we directly compared the antiviral efficacy of Ampligen and poly ICLC, two polyribonucleotides (Table 10). In addition, we also tested two different Ampligen preparations. The results showed that both Ampligen preparations and poly ICLC provided complete protection in repeated early therapeutic treatment schedules at doses as low as 0.5 mg/kg. However, delaying treatment until 24 hr after infection resulted in a complete loss of activity from all compounds.

We have previously shown that SFV is sensitive to rHuIFN-A or rMuIFN-G in a day 0 to +6 treatment schedule (65). However, when given as a single therapeutic dose (4 hrs after infection) rHuIFN-A, but not rMuIFN-G, retained antiviral activity. During this year we have shown that, similar to rHuIFN-A, rMuIFN-B is also effective when given as a single early therapeutic dose (Table 10). When repeated therapeutic treatment with IFN was delayed to day +1 or day +2 after infection, the antiviral activity of rMuIFN-B and rMuIFN-G was completely lost, while rHuIFN-A retained some antiviral activity at a 10,000 U dose.

Preliminary work on the effectiveness of GLA-60, a synthetic analogue of lipid A, indicated this compound, when administered at 1.5 mg/kg 3 days before and on the day of infection, had good antiviral activity (Table 10). Reducing the dose to 0.5 mg/kg reduced, but did not eliminate the antiviral activity of GLA-60. However, when administered as a single prophylactic dose, GLA-60 was ineffective at either a 0.5 mg/kg or 1.5 mg/kg dose.

In two experiments with rHuCSF-M, a small but significant increase in the MST was observed in a day -4 to day 0, but not a day -4 to day +2, treatment schedule (Table 10).

Antiviral efficacy of immunomodulators against Caraparu bunyavirus. As we have previously reported (64,65), Caraparu virus infection continued to be the least sensitive infection model to immunomodulator treatment (Table 11). In contrast with our results against SFV, where poly ICLC and Ampligen were equally effective, only poly ICLC at 1 mg/kg provided excellent protection against Caraparu virus when administered from day -1 to day +6 after infection. Either of the Ampligens administered at 4 mg/kg in the same treatment schedule were only partially effective.

In contrast with their efficacy against the other three viruses, rHuIFN-A and rMuIFN-B were only slightly effective against Caraparu virus in combined repeated daily prophylactic and therapeutic treatment schedules (Table 11). rMuIFN-G has exerted consistent partial antiviral activity against Caraparu virus infection, in a day -1 to day +6 treatment regimen or when initiation of treatment was delayed until 4 hrs post infection. This activity, however, was lost if treatment was further delayed until 2 days after infection.

We have previously reported that the nucleoside analog ribavirin (100 mg/kg) was effective against Caraparu virus in repeated treatment schedules beginning on either day -1 or day 0 (65). In this year of the contract we have shown that the start of treatment could be delayed to day +1, but not day +2, with no loss in efficacy (Table 11). A newer form of the drug was tested and this also appears to have good antiviral activity against Caraparu virus (Table 11).

A variety of combination treatments against Caraparu virus have also been tested. Combined treatment with ribavirin (100 mg/kg on days +2 to +6 and on days +8, +10, +12, +14, and +16) and rMuIFN-G (500 U on day 0 to day +6) had good synergistic activity against Caraparu virus (Table 12). Delaying the start of rMuIFN-G treatment until day +2 produced no significant synergistic effect even when the dose of rMuIFN-G was increased two-fold or treatment was extended to day +8. Other combination treatments with Ampligen + poly ICLC, ribavirin + poly

ICLC, and poly ICLC + rMuIFN-B produced no synergistic activity against Caraparu virus infection.

Selective Depletion of Interferon with Anti-interferon Serum and Effects on Antiviral Resistance Induced by Immunomodulators

A long-standing question has been whether interferon (IFN) could be a common mechanism of action for the diverse immunomodulators that have shown antiviral efficacy. Although some very effective immunomodulators induce no or very low levels of detectable circulating IFN (11,25,26,56,57), there has always been the possibility that these immunomodulators might induce protective IFN localized at the critical sites of infection. Our previous cell depletion studies with 89Sr (61) or DMDP liposomes (65,73), would not rule out the possibility that a decrease in resistance might be due to IFN induction by the depleted cell. Therefore, we performed several studies in this last year to assess directly the role of IFN in the antiviral activity of several of our most effective immunomodulators.

These studies had the following parameters:
(i) Use of SFV infection model. The pathogenesis course of this virus is rapid, with a median time of death of 4-5 days, so that one treatment with anti-IFN should remain effective throughout the course of infection. We and others have also shown that SFV infection is very sensitive to the prophylactic use of immunomodulators.
(ii) Single prophylactic treatment with effective doses of the immunomodulators. We began with prophylaxis, since most of the immunomodulators are most effective by this regimen. Depending upon the results, we may later determine the effects of anti-IFN serum on therapeutic treatment with immunomodulators, in order to determine whether IFN is involved in antiviral effects by both regimens.
(iii) Treatment i.p. with a single dose of anti-IFN 4 hr before i.p. treatment with the immunomodulators, in order to enhance the interaction between any IFN induced and the anti-IFN serum. The anti-IFN serum was produced in sheep against NDV induced murine IFN that is 70% beta and 30% alpha IFN, and has been used successfully in a number of *in vivo* studies (5,45). Treatment was with a dose of anti-IFN serum sufficient to neutralize all IFN induced. Corresponding controls were inoculated with normal sheep serum (NSS)

Characterization of the effects of anti-IFN serum on interferon in vitro and in vivo. Before the experiments could be performed, preliminary information was essential concerning: (i) what level of alpha/beta IFN the various immunomodulators induce, (ii) what level of alpha/beta IFN does SFV infection induce, (iii) what level of anti-IFN serum can be used without markedly affecting the pathogenesis of SFV infection, and (iv) what is the *in vitro* and *in vivo* neutralizing activity of the anti-IFN serum for various types of IFN.

When IFN levels were measured at 6, 12, 18, 24 and 48 hr after SFV infection, no detectable levels of IFN were found in either the plasma or peritoneal fluids. In order to determine whether anti-IFN serum affected markedly the lethality of SFV infection, a small LD_{50} curve was performed in mice treated with the control NSS or anti-IFN serum. There was no significant change in LD_{50} with concentrations of anti-IFN ranging from 10,000 to 100,000 neutralizing

units, although the mice generally died a few hours earlier.

Table 13 shows that only treatment of mice with poly ICLC induced high levels of plasma IFN (peak of 2900 U at 6 hr), although Ampligen induced 135 U/ml at 3 hr. Minimal levels of IFN were also detectable in the peritoneal fluids of mice in the poly ICLC and the rHuIFN-A A/D groups.

The in vitro neutralizing activity of anti-IFN or NSS against immunomodulators was then evaluated. The ability of the anti-IFN serum to neutralize plasma IFN induced by poly ICLC, rHuIFN-A A/D, and a NDV induced plasma alpha/beta murine IFN was assessed in vitro (Table 14). These results indicate that anti-IFN serum very efficiently neutralizes natural murine alpha/beta IFN and poly ICLC induced IFN, and that the neutralizing units administered were accurate. Based upon these data, theoretically 3000-5000 neutralizing units of anti-IFN serum should be sufficient to neutralize the poly ICLC induced IFN, and much less should be effective against the other immunomodulators that did not induce detectable levels of IFN.

The anti-IFN serum was much less effective in neutralizing rHuIFN-A A/D; the neutralizing activity was at least 10 times less than that observed against the other IFNs. Thus, to neutralize 10,000 U of rHuIFN-A A/D, at least 100,000 neutralizing units of anti-IFN would need to be administered. The lowered activity of anti-murine alpha/beta IFN serum against rHuIFN-A A/D is not surprising for two reasons. First, rHuIFN-A A/D is a human IFN, and as such, does not have complete antigenic cross reactivity with murine IFN. Secondly, rHuIFN-A A/D is completely alpha IFN, whereas the murine anti-IFN serum is directed primarily against beta IFN.

The in vivo neutralizing ability was measured in three experiments by inoculating mice with NSS or anti-IFN serum, then administering the immunomodulators, and measuring plasma IFN levels at various periods thereafter (Table 15). These data indicated that the in vitro neutralizing activity did not completely mimic the in vivo conditions. Administration of 50,000 neutralizing units of anti-IFN serum appeared to decrease the plasma titer of rHuIFN-A A/D. However, administration of even 100,000 neutralizing units of anti-IFN serum did not completely reduce the plasma IFN titers produced by poly ICLC administration. Therefore, although 10,000 neutralizing units of anti-IFN should be sufficient to neutralize any IFN from immunomodulators that induced less than 50 U, it might not be sufficient to completely neutralize IFN induced by poly ICLC. Thus, experiments were performed with concentrations of anti-IFN ranging from 10,000 to 100,000 neutralizing units.

In vivo effects of anti-IFN serum on immunomodulator induced resistance to SFV infection. Table 16 illustrates the results when 2000 or 10,000 neutralizing units of anti-IFN were used. The anti-IFN treatment reduced the antiviral efficacy of ABMP, but had no effect on any of the other immunomodulators tested. When the concentration of anti-IFN was increased to 50,000 neutralizing units (Table 17), the antiviral activity of ABMP was also reduced. In addition, the antiviral efficacy of Ampligen was reduced; whereas mice treated with Ampligen and NSS showed 0% mortality, those treated with Ampligen and anti-IFN showed 50% mortality. When the concentration of anti-IFN serum was increased to 100,000 neutralizing units, the antiviral activity of most

of the immunomodulators -- Ampligen, MVE-2, ABMP and CL246,738 -- was reduced (Table 18). However, the antiviral activity of poly ICLC and rHuIFN-A A/D was not affected.

These results emphasize the complex role that IFN induction may have in the antiviral activity of these immunomodulators, as well as the difficulty in properly interpreting depletion studies.

(i) For some immunomodulators, such as ABMP and Ampligen, the in vivo antiviral activity was neutralized relatively efficiently by anti-IFN serum treatment. Thus, it is likely that much of the antiviral activity of ABMP and Ampligen may be ascribed to induction of IFN, even though none or very low levels of circulating IFN were observed. These data suggest that local concentration of IFN at critical sites may be more reflective of the action of these immunomodulators than any levels of circulating IFN.

(ii) There was not a clear correlation between in vitro and in vivo neutralizing activity for other immunomodulators, such as poly ICLC. Anti-IFN serum effectively neutralized poly ICLC induced IFN in vitro; however, it did not diminish the antiviral effectiveness of poly ICLC in vivo, even when 100,000 neutralizing units of antibody were used (33 times the amount sufficient to neutralize the peak 3000 units measured after poly ICLC). These data suggest that mechanisms in addition to IFN may be involved in the antiviral activity of poly ICLC. This may explain the greater antiviral activity of poly ICLC against Caraparu virus as compared with Ampligen (Table 11).

(iii) For certain immunomodulators, such as MVE-2 and CL246,738, it apparently required over 2000 times more neutralizing antibody as the peak detectable IFN titer (< 50 U) to decrease antiviral activity. These data support the hypothesis that mechanisms other than IFN may be responsible for most of the antiviral activity of MVE-2. Alternatively, single administration of anti-IFN serum 4 hr before inoculation of MVE-2 or CL246,738 may not provide sufficient neutralizing antibody at the time (18-20 hr) of peak IFN titers induced by these immunomodulators. Repeated administration of anti-IFN serum is planned to answer this question.

(iv) The antiviral activity of rHuIFN-A A/D was not affected at all by treatment with 100,000 neutralizing units of anti-IFN. These data suggest that the antiserum, directed primarily against murine beta IFN, had little cross reactivity with human alpha IFN in regard to inhibiting antiviral action. Interestingly, the same antibody has been shown to be effective in vivo in decreasing the splenic NK cell activation properties of rHuIFN-A A/D. These differences may reflect neutralization efficiency in the spleen versus other sites after the i.p. injection of the antiserum.

The results emphasize the complexities involved in interpreting IFN depletion studies, even after characterizing the overall effects of the strategy. Thus, independent experimental approaches would be helpful in confirming these results. We are attempting to obtain monoclonal antibodies against murine alpha and beta IFNs that are effective in neutralizing IFN in vivo.

DISCUSSION OF THIRD CONTRACT YEAR

Our comparative analysis of the antiviral effectiveness of immunomodulators has continued to emphasize the ability to provide broad spectrum antiviral activity against HSV-2, Banzi flavivirus, Semliki forest alphatogavirus, and Caraparu bunyavirus. As we have previously noted (65,75), there were distinct patterns in sensitivity of these viruses to the different immunomodulators. HSV-2, Banzi virus and SFV infections were more sensitive to treatment with immunomodulators than was Caraparu virus infection.

Of particular importance were the findings that various therapeutic treatment schedules with immunomodulators were effective. The HSV-2 infection was most sensitive; therapeutic treatment with Ampligen or any of the three types of IFN was effective even when the start of treatment was delayed until 48 hr after infection. Against the Banzi virus model infection, Ampligen, poly ICLC, and the three IFNs were active in repeated therapeutic treatment schedules initiated as late as 24 hr after infection. These compounds were also effective against SFV in a repeated early therapeutic treatment schedule beginning 4 hr after infection; however, all except rHuIFN-A were ineffective if treatment was delayed to 24 hr post infection. In contrast with their effectiveness against the other viruses, therapy initiated early (day 0) with rHuIFN-A or rMuIFN-B was not effective against Caraparu virus while rMuIFN-G provided significant protection.

Of interest was the finding that poly ICLC, but not Ampligen, gave excellent protection against Caraparu virus infection in a repeated prophylactic/therapeutic treatment schedule. This is surprising since both compounds have similar structures and are believed to act through the induction of alpha/beta IFN (28,29,51). These results suggest that (i) poly ICLC has direct antiviral activity against Caraparu virus, (ii) poly ICLC induces antiviral activity through mechanisms in addition to IFN, or (iii) poly ICLC induces higher levels of IFN over a longer period of time in the critical target organs.

There are several possible explanations for the finding that, of the three IFNs, rMuIFN-G demonstrated the best antiviral activity against Caraparu virus infection. First, the immunomodulatory effects induced by rMuIFN-G may be more crucial to limiting Caraparu virus than those induced by rHuIFN-A or rIFN-B. Wilson and Westall (95) showed that of the three IFNs, gamma IFN was the most potent activator of human adult and neonatal Mϕ. Many other studies have also indicated gamma IFN as a powerful Mϕ activator (68,69,75,89). Therefore, it is possible that Caraparu virus infection may be more sensitive to Mϕ involvement than Banzi virus or SFV. Second, while all three classes of IFN augment NK activity (17,75) gamma IFN appears to have a different mechanism of NK activation. This is evidenced by the recent studies of McGinnes et al., (54) showing that alpha and gamma IFN induced different conformational changes in the NK cell membrane and Gustafsson et al., (27) showing that alpha IFN, but not gamma IFN, induced the synthesis of various proteins in the NK population. It is therefore possible that the different IFNs induce NK cells with different antiviral states or effector functions. Alternatively, different viruses may vary in their sensitivity to different cells in the heterogeneous NK cell population (91). Third, the different pathogenesis patterns of the viruses might also be important. Our findings with Caraparu virus infection indicate that the virus

replicates in the liver and causes death through hepatic cell damage, whereas Banzi virus and SFV appear to cause death through CNS destruction (19,35). Caraparu virus also causes death more quickly than does Banzi (4 days as compared with 6 to 8 days). Caraparu virus infection may therefore cause disease so fast that normal immune responses have no chance to develop. Gamma IFN, being a potent and rapid activator of Mϕ and NK cells, may induce an effective early defense mechanism in liver Mϕ and NK cells.

Immunomodulators appear to exert their protective effect early in infection, before the virus has established a productive infection or has become sequestered in a privileged site such as the CNS (62,75). During the early stage of infection natural defense mechanisms, such as Mϕ and NK cells, are most important in limiting infection (63,91). We have previously shown that most immunomodulators induce varying degrees of Mϕ and NK cell activation (75). Thus, it is tempting to propose Mϕ and NK cell involvement in the observed antiviral activity. It is possible, however, that immunomodulators may have direct antiviral effects or that they control virus proliferation through induction of mediators, such as IFN, that interfere with virus replication. To directly assess the contribution that macrophages and NK cells have in immunomodulator-induced antiviral activity, we have combined immunomodulator treatment with selective cell depletion methods. For example, we have shown that treatment of CD-1 mice with ^{89}Sr, which depletes circulating leukocytes and NK cells while leaving tissue Mϕ intact, does not alter immunomodulator induced resistance to HSV-2 (61). Anti-NK 1.1 antibody treatment, which selectively depletes NK cells, has been used to demonstrate the involvement of NK cells in natural and IFN enhanced tumor resistance (44,78). This antibody, unfortunately, proved to be unable to reduce NK cells in the B6C3F1 model used in our studies.

Another central question that we have investigated this year is whether IFN induction by the various immunomodulators is a common mechanism of action. To address this we have studied the effect of anti-IFN antibody treatment on the antiviral efficacy of a number of immunomodulators against lethal SFV infection. Our results indicate that IFN induction may be an important component of the antiviral activity of Ampligen, ABMP, and to a lesser extent CL246,738 and MVE-2. These data extend those recently reported for CL246,738 where both alpha and beta IFN were found to be involved (76,90). IFN has previously been reported to be important for the antiviral activity of the pyrimidinones from correlative studies with IFN titers (52). We have previously viewed the antiviral activity of MVE-2 as being independent of IFN, because there was no correlation of circulating IFN titers with antiviral activity, and depletion of IFN with a lower titered antiserum (sufficient to neutralize IFN in vitro) did not reduce antiviral activity against EMC virus (61). The present studies, however, using extraordinarily high titers of anti-IFN serum, reveal that IFN may be a component of the antiviral activity of MVE-2, at least against SFV infection. There was, however, no clear correlation between the in vitro neutralization activity of the anti-IFN antibody and reduction of in vivo antiviral activity by poly ICLC, suggesting that mechanisms in addition to IFN may be involved in the antiviral efficacy of poly ICLC. These results, obtained with single administration of anti-IFN, were recently validated with two administrations of 100,000 neutralizing units of anti-IFN serum with each injection (unpublished observations).

The overall results of our selective depletion studies emphasize the complexity of the action immunomodulators may have in vivo. It is apparent that considerable systematic characterization needs to be performed before results with studies using such selective depletion methods can be interpreted clearly. Nevertheless, the combination of selective depletion methodology, coupled with correlative studies of the antiviral activity of IFN, Mϕ and NK cells, are powerful experimental tools for establishing whether the antiviral activity of immunomodulators involves or requires IFN, Mϕ or NK cells.

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SUMMARY OF RESULTS OF THE ENTIRE CONTRACT PERIOD

Immunomodulator Profiles

Broad spectrum intrinsic antiviral resistance of peritoneal Res Mϕ and Res KC. Res peritoneal Mϕ are relatively nonpermissive for a broad spectrum of viruses, as evidenced by no or very low production of infectious virions and no cytopathic effect (Table 19) (48,82). The limited studies performed with KC indicate that these Mϕ are equally or more resistant to infection with the viruses tested (HSV and Pichinde). Some viral gene expression does occur in Res Mϕ infected with HSV-1 or HSV-2 (64,82), and it is possible that some gene expression or virus persistence may occur with the RNA viruses as well. These questions, although important, were outside of the contract workscope.

Intrinsic antiviral activity of immunomodulator activated peritoneal Mϕ and KC. Immunomodulator activated peritoneal Mϕ and KC also exhibited marked intrinsic antiviral resistance to HSV and Pichinde virus. There was a trend towards enhanced intrinsic antiviral activity of activated peritoneal Mϕ, but, because Res Mϕ are already so resistant, it was difficult to establish quantitative differences between the Mϕ types. Additional data are needed to determine whether there are significant differences in the degree of antiviral resistance between Res Mϕ or immunomodulator activated Mϕ.

C. parvum activated peritoneal Mϕ were equally resistant as Res Mϕ to replication of HSV, but showed considerable cytopathic effect after HSV infection. None of the other Mϕ tested showed significant cytopathic effect after infection with HSV, Pichinde or SFV. Infection of Res or immunomodulator activated peritoneal Mϕ with Pichinde virus did cause a definite increase in the spreading of the cells. This increased spreading required live virus since UV inactivated Pichinde virus did not cause the effect.

Extrinsic antiviral activity of Res and immunomodulator activated peritoneal Mϕ. In addition to their intrinsic ability to inhibit a broad spectrum of viruses, Res and most immunomodulator activated Mϕ exhibited significant extrinsic antiviral activity against HSV and Pichinde, but less toward SFV, when these viruses were replicating in permissive cells. The activity with immunomodulator activated Mϕ was seldom significantly different from that exhibited by Res Mϕ alone, but there was a trend for enhanced antiviral activity. More data need to be accumulated before we can conclude definitively whether any of the immunomodulator activated Mϕ exhibit a significant increase in extrinsic antiviral activity as compared with Res Mϕ.

Summary of immunomodulator profile data. A summary of our findings in regard to immunomodulation of nonspecific immune responses by antivirally active compounds is shown in Table 20. The activities were measured on what would have been the first day of viral infection after prophylactic treatment with the compounds. Most of the immunomodulators that were tested by a prophylactic regimen activated NK cell cytotoxicity and activated Mϕ to at least some degree. However, no single nonspecific immune parameter that was measured could be correlated consistently with antiviral activity. For example, MVE-2 and C. parvum are among the most active compounds in regard to in vivo antiviral activity, yet they induced none to very low levels of IFN activity or NK cell cytotoxicity by

the treatment schedule used (6,11,55,57). Likewise, the variations in degree of peritoneal Mϕ activation do not appear to correlate with differences in degree of antiviral activity.

Overall conclusions:

(i) Most immunomodulators have pleiotropic effects in vivo, inducing changes in several nonspecific immune system functions.

(ii) There may be no unifying mechanism of antiviral action. Because each immunumodulator tested has pleiotropic effects on nonspecific immune parameters, protection against each virus infection may involve different aspects of host resistance and an increase in any one of these may produce increased resistance.

(iii) Nonspecific immune activities other than the ones measured may be involved in antiviral protection. We may not yet have identified the unifying immune parameter. For example, the ability of an immunomodulator to activate peritoneal cells for antitumor activity may not be central to its antiviral activity. This issue can best be addressed by determining the effects of depletion of various cell types on antiviral resistance and the efficacy of the immunomodulators.

(iv) Antiviral activity of cells other than those investigated may be primary in antiviral resistance. For example, liver Kupffer Mϕ may be the most relevant cell in regard to antiviral activity for infection with Caraparu virus which targets to the liver.

Kupffer Cell (KC) Isolation and Characterization

We have been unable to perform the KC-virus experiments as originally proposed because of unexpected difficulties in removing liver endothelial cell (LEC) contamination from our KC preparations. Since the primary emphasis of this contract was on antiviral and immunomodulator profiles, we had only one person working on this part of the project at 30-50% effort. Due to the technical difficulties found during our work with mouse KC, and the small number of cells recovered per mouse, we now realize that one or more full time people are needed if multiple groups of livers from immunomodulator and control animals are to be processed in a day.

It is evident from our work and that of others that there is still no standard successful separation procedure that has been developed for isolation of mouse KC. Most of the KC literature deals with the rat system. It is now clear that straightforward modifications of the procedures used with rat KC is not sufficient, especially when 10 or more mouse livers need to be processed at one time in order to obtain sufficient cells for experimentation. The standard procedure in the rat is to digest the liver in situ, by perfusing the liver with warm collagenase or pronase for 15-30 min, followed by differential centrifugation, density gradient centrifugation or elutriation. Such an in situ digestion procedure in the mouse is not routinely used because the total number of NPC recoverable from a mouse liver is only about 20% of what is recoverable from the much larger rat liver. One has to process 10-20 mouse livers to get the same number of cells recovered from 2-4 rat livers. Using the long perfusion procedure, this would require 2-4 hr merely to obtain the mouse liver cells,

prior to the several hr procedure to separate NPC from liver parenchymal cells. Separation procedures in mice usually have used short term in situ perfusion to remove blood (3-5 min), followed by enzymatic digestion in vitro, and subsequent separation of KC using density gradient centrifugation with one of several media (4,16,18,43 72), and/or centrifugal elutriation (43). In most of these papers, small numbers of cells were sufficient for experimentation. We have found that isolation procedures useful for 1-5 mice cannot be applied easily to groups of 10 or more.

Another difficulty peculiar to mouse KC is that, in the mouse there is no standard way of identifying cells as KC (positivity for peroxidase is generally used in the rat) (20,43,71,86) but is not as reliable in the mouse. For instance, some researchers have used nonspecific esterase positivity as a criteria for KC (4,70), while others have used Fc receptor rosetting (16) or nonspecific phagocytosis (22). These criteria, however, must be used with caution because they are also characteristic of LEC (71). We have questioned the true identify of the non phagocytic cells in our culture because endothelial cells are reported to stick poorly to untreated surfaces and are often difficult to maintain in culture. Consequently, we have spent a great deal of time assessing LEC contamination in our NPC fraction. We have approached this problem by monitoring LEC numbers using various LEC specific markers and KC numbers using KC specific markers.

Overall conclusions:

(i) The NPC fraction obtained after short term in situ perfusion, followed by in vitro digestion with collagenase and metrizamide gradient centrifugation contains the following cell populations and characteristics (Table 21). The adherent population after 24 hr consists of 65% KC with contaminating LEC, and these cells have the following characteristics (Table 21).

(ii) The population of KC can be significantly enriched, (less LEC contamination and cell debris) by the use of one of several additional purification steps. However, none of these has provided completely satisfactory results. The most promising appears to be C3B receptor mediated rosetting.

(iii) Resident and immunomodulator activated KC, although studied in a few experiments, exhibit marked intrinsic resistance to replication of HSV and Pichinde viruses, comparable or greater than that exhibited by resident peritoneal Mϕ. These data suggest that resident and immunomodulator activated peritoneal Mϕ and KC both have marked antiviral resistance. There may be significant differences in the mechanisms involved in the antiviral resistance, which remain to be elucidated.

Broad Spectrum Antiviral Efficacy of Treatment of Mice with Immunomodulators

Antiviral Efficacy of Immunomodulators against HSV-2. HSV-2 infection was the most sensitive of our in vivo models. Prophylactic treatment with either of the two positive control immunomodulators, C. parvum and MVE-2, or with CL246,738 down to 25 mg/kg, provided complete protection. Single prophylactic treatment with the pyrimidinones ABPP and AIPP, but not ABMP, was also effective. The mismatched polyribonucleotide Ampligen was shown to be quite effective upon repeated therapeutic treatment beginning as late as 2 days after infection. Similarly, when the start of repeated therapeutic treatment with any of the three IFNs was delayed to day +2 after infection they still exerted excellent antiviral activity. Single experiments testing treatment of mice with rHuTNF-A or rHuCSF-M did not provide antiviral activity against HSV-2 infection.

Antiviral Efficacy of Immunomodulators against Banzi virus. Like HSV-2, infection with Banzi virus was also a sensitive model in which to evaluate immunomodulators. Single prophylactic administration with C. parvum, MVE-2, or Cl246,738 down to at least 12.5 mg/kg produced complete protection from mortality. Delaying treatment with CL246,738 until 24 hr after infection removed almost all antiviral activity. The start of repeated therapeutic treatment with Ampligen or poly ICLC, however, could be delayed until 48 hr after infection without a complete loss of antiviral activity. Results with the recombinant IFNs indicated that therapeutic treatment with rHuIFN-A or rMuIFN-B was considerably more effective against Banzi virus than was therapeutic treatment with rMuIFN-G. With the pyrimidinones, ABPP, but not ABMP, was effective against Banzi virus infection in single prophylactic, single therapeutic, and repeated therapeutic treatment schedules. As with HSV-2 infection, rHuTNF-A and rHuCSF-M were ineffective against Banzi virus infection.

Antiviral Efficacy of Immunomodulators against SFV. Parenteral infection with this alphatogavirus also provided a sensitive model in which to evaluate immunomodulators. Single prophylactic treatment with C. parvum, MVE-2, CL246,738, Ampligen, or any of the three pyrimidinones was completely effective against lethal SFV infection. Repeated early therapeutic treatment with Ampligen or poly ICLC provided complete protection against SFV; however, delaying the start of treatment until 24 hr after infection resulted in a complete loss of activity. SFV infection was sensitive to early therapeutic treatment with all three classes of IFN, but only rHuIFN-A retained some antiviral activity in a repeated therapeutic treatment schedule beginning 24 hr after infection. Repeated prophylactic/therapeutic treatment with the bacterial cell wall preparations MPL + TDM + CWS provided excellent antiviral activity. GLA-60, a synthetic lipid A analog, had good antiviral activity in repeated, but not single, prophylactic treatment.

Antiviral Efficacy of Immunomodulators against Venezuelan Equine Encephalitis Virus. Single prophylactic treatment with MVE-2 or with CL246,738 down to at least 100 mg/kg provided good protection against VEE. Single prophylactic treatment with C.parvum or Ampligen was not effective against VEE. However, combined prophylactic/ therapeutic treatment with Ampligen was very effective, and repeated therapeutic treatment was moderately effective against

VEE. Repeated therapeutic, but not single therapeutic, treatment with rHuIFN-A was effective against lethal VEE infection. rMuIFN-G was only effective against VEE when given in a repeated and combined prophylactic/therapeutic treatment schedule.

Antiviral Efficacy of Immunomodulators against Caraparu virus. Caraparu virus infection was the most insensitive to treatment with immunomodulators. In contrast with their effectiveness against the other viruses, only moderate protection against Caraparu virus was produced with MVE-2 or CL246,738. Unlike our results with SFV, where poly ICLC and Ampligen were equally effective, only poly ICLC provided excellent protection against Caraparu virus in a day -1 to day +6 treatment schedule. Like Ampligen, rHuIFN-A, and rMuIFN-B were not very effective against Caraparu virus in repeated prophylactic/therapeutic treatment schedules. rMuIFN-G, however, did exert consistent partial antiviral activity against Caraparu virus when given from day -1 through day +6 after infection. The nucleoside analog ribavirin was highly effective against Caraparu virus in repeated treatment schedules beginning on either day -1, day 0, or day +1. Although the effectiveness of ribavirin was lost when treatment was delayed to day +2, combination treatment at this time with rMuIFN-G resulted in good synergistic activity. Other combination treatments, however, with Ampligen and poly ICLC, ribavirin and poly ICLC, and poly ICLC and rMuIFN-B produced no synergistic activity against Caraparu virus infection.

Overall conclusions.

(i) Systemic i.p. infection with Banzi, SFV, or HSV-2 provide sensitive systems in which to evaluate the antiviral efficacy of immunomodulators. Infection with the alphatogavirus, Venezuelan equine encephalitis, is somewhat less sensitive, and infection with the bunyavirus, Caraparu virus, is much less sensitive to immunomodulators.

(ii) Most, but not all, of the immunomodulators tested provided significant broad spectrum antiviral efficacy when administered in either a prophylactic regimen, or in a repeated prophylactic + therapeutic regimen (Table 22). The active immunomodulators included: C. parvum, TDM, MPL + TDM, CWS + MPL + TDM, CL246,738, ABPP, AIPP, ABMP, MVE-2, Ampligen, poly ICLC, GLA-60, rMuIFN-G, rHuIFN-A A/D, rMuIFN-B.

(iii) The inactive immunomodulators included: PA-PE, GE-132, HDPP, S-209, rHuTNF-A and rHuCSF-M. It should be noted, however, that these "inactive" immunomodulators were not tested in a wide variety of doses and regimens.

(iv) All three types (alpha, beta, gamma) of recombinant IFNs provided significant therapeutic antiviral activity in at least one of the model infection systems. There were, however, differences in sensitivity to therapeutic treatment with the three IFN types. All three IFN types were equally effective against HSV-2 infection, alpha IFN was slightly more effective than the others against SFV infection, while only alpha and beta IFN were effective against Banzi virus infection, and only gamma IFN was consistently effective against Caraparu virus infection.

(v) Synergy between gamma IFN and ribavirin, administered therapeutically, could be demonstrated for Caraparu virus infection.

No synergy was evident with ribavirin + poly ICLC, or poly ICLC combined with either Ampligen or beta IFN.
(vi) Ampligen and poly ICLC, although both are double stranded RNA preparations, differ considerably in antiviral efficacy. Poly ICLC was clearly more effective, providing protection against Caraparu virus infection which is completely insensitive to therapeutic treatment with Ampligen.

Selective Depletion of Tissue Mϕ and NK Cells and Interferon

Four experimental procedures for selective depletion in B6C3F1 mice were evaluated during this contract. We spent most of our time in the last year on evaluating the effects of anti-IFN serum, because of the major question whether IFN is the common mechanism involved in the antiviral activity of immunomodulators.

Effect of Mab to NK1.1 antigen on NK cell activity in the B6C3F1 mouse. The IgG2a Mab NK1.1, has been used quite successfully to deplete neonatal and adult mice of several strains of their NK cells, and to decrease tumor resistance (44,78). Its effects on antimicrobial host resistance, however, have not been evaluated. Our experiments with anti NK1.1 Mab treatment of the adult B6C3F1 mouse, however, proved it to be ineffective in this mouse strain. Neither spontaneous nor poly I:C (IFN) inducible NK cell activity was sufficiently reduced, even when (i) the dose of the antibody was increased from the recommended 50μl/mouse to 200μl/mouse, (ii) mice were pretreated with the IFN inducer, poly I:C, prior to treatment with Mab NK1.1 to increase the number of target antigens, or (iii) mice were treated repetitively with Mab NK1.1 every 10 days. It is apparent that this Mab is relatively ineffective in the adult B6C3F1 mouse, especially against IFN enhanced NK cell activity. For this approach to be useful either a different mouse strain will have to be used, or treatment initiated in neonatal life and continued weekly thereafter. Therefore, effort on this approach was stopped.

Effect of i.v. inoculation of the toxin DMDP encapsulated in liposomes on Mϕ, NK cells and host resistance. The toxin dichloromethylene diphosphonate (DMDP) has profound depleting effects on splenic and liver Mϕ (88) and humoral immune functions in these organs (12); however, its effects on host resistance have not been determined. The DMDP depletion system is particularly interesting, because it is reported to selectively deplete for about one week splenic and liver Mϕ, two of the most important tissue Mϕ populations without affecting other tissue Mϕ compartments.

We established that two i.v. injections of DMDP liposomes (days -4 and -2 prior to assay) decreased resistance of mice inoculated with HSV-2 or Listeria monocytogenes (73), associated with a marked but transient (1-3 days) decrease in splenic NK cell activity, a marked leukocytosis of lymphocytes and PMN (65) that persisted for about 6 days, and no significant effect on peritoneal cell populations or on peritoneal Mϕ ectoenzyme profiles (65). Interestingly, there was no pronounced monocytosis. The fact that the increase in circulating leukocytes occurred so rapidly (by 1 day), and the fact that the cells mostly appeared to be mature, argues against the leukocytosis being due to enhanced

maturation of bone marrow stem cells. We speculate that the leukocytosis is related to an outflow of the large circulating pool of lymphocytes and PMNs that is contained within the spleen and liver, due to destruction of the tissue Mϕ and subsequent alterations in organ architecture. Cytochemistry on frozen spleen sections for the presence of acid phosphatase and the F4/80 Mϕ antigen confirmed the loss of splenic Mϕ after double i.v. treatment (van Rooijen, unpublished observations).

The data showing no change in the peritoneal Res Mϕ population (65) after i.v. administration of DMDP in multilamellar liposomes, which cannot penetrate capillaries and gain entrance into the peritoneum, provide additional support for the concept that peritoneal Mϕ are separated from at least several other Mϕ compartments in the body (79).

Effect of i.p. treatment of mice with ricin encapsulated in liposomes. As a counterpoint to the i.v. toxin liposome experiments, CD-1 mice were inoculated i.p. with ricin liposomes (collaboration with Dr. Alvin Volkman, East Carolina University School of Medicine). The toxin was inoculated into normal mice and into mice that had been treated with 89Sr to deplete the mice of monocytes and any inflammatory exudate of monocyte-Mϕ to the peritoneal cavity after ricin liposome treatment (81). The four groups of mice were then infected i.p., 24hr after ricin liposome treatment, with Listeria monocytogenes. Treatment of either control or 89Sr treated mice with ricin liposomes caused a marked reduction in resistance to Listeria (65).

Thus, we now appear to have two distinctly different methods with which to selectively deplete mice of the splenic and liver Mϕ populations, or the peritoneal Mϕ population, without affecting the other compartment. The issues now to be addressed are to: (i) demonstrate the lack of effect on the respective tissue Mϕ compartments (peritoneal versus spleen and liver) by isolating KC and peritoneal Mϕ and (ii) whether immunomodulator treatment can overcome such depletion of peritoneal, or of splenic and liver Mϕ and the associated decrease in natural resistance. The completion of these experiments was delayed in order to investigate an experimental question that was not included in the original contract but was deemed to be important to resolve, namely the question of whether IFN is the common mechanism of the antiviral activity of immunomodulators.

Effect of anti-IFN serum on natural and immunomodulator induced antiviral resistance against SFV infection. Much of the antiviral activity of ABMP and Ampligen may be ascribed to induction of IFN, because anti-IFN serum relatively efficiently reduced the antiviral activity. The antiviral activity of MVE-2 and CL246,738 was also reduced by anti-IFN treatment, but this required single treatment with > 2000 times more neutralizing units of antibody as the peak detectable IFN titer (< 50 U). Since these immunomodulators induced none or very low levels of circulating IFN, the data suggest that local concentrations of IFN may be most important in antiviral resistance. In contrast to these immunomodulators, mechanisms in addition to IFN may be involved in the antiviral activity of poly ICLC. This may explain the greater antiviral activity of poly ICLC as compared with Ampligen. There was not a clear correlation between in vitro and in vivo neutralizing activity for poly ICLC. Anti-IFN serum effectively neutralized poly ICLC induced IFN in vitro, but did not diminish the

antiviral effectiveness of poly ICLC *in vivo*, even when 33 times the amount which was needed to neutralize the peak IFN levels was given.

Overall conclusions.

(i) The DMDP liposome system is now ready for use in assessing the contribution of splenic and liver Mϕ, and perhaps NK cells, to immunomodulator induced antiviral resistance. The data demonstrate the usefulness of this method for selective depletion of splenic and liver Mϕ without affecting peritoneal Mϕ, as well as point out the complexity of any of the available depletion methods for Mϕ since splenic NK cells were depressed as well as splenic Mϕ. Thus, it is necessary to characterize completely the depletion system in order to interpret changes found in host resistance.

(ii) Our results emphasize the complex role that IFN induction may have in the antiviral activity of immunomodulators. IFN induction appears to be a major component of the antiviral effectiveness of Ampligen and ABMP, and perhaps of MVE-2 and CL246,738, but IFN appears to be only one component of poly ICLC induced resistance. Independent experimental approaches, using the cell depletion methods described above as well as monoclonal antibodies against alpha and beta IFN, will be helpful in delineating the role in antiviral resistance played by IFN and the cells that produce that IFN.

(iii) Monoclonal antibody NK 1.1 is not effective in the adult B6C3F1 mouse in reducing NK cell activity, particularly IFN enhanced activity. Other monoclonal antibodies, or other procedures with NK 1.1 (e.g. treatment of neonatal mice) will have to be used to assess the contribution of NK cells to immunomodulator induced antiviral resistance.

RECOMMENDATIONS FROM THE ENTIRE CONTRACT PERIOD

1. We recommend that work with immunomodulators as antiviral agents continue to be developed for independent and combination therapy against viral infections. Our work, and that of others, has clearly established that a wide variety of diverse immunomodulators can produce marked, broad spectrum antiviral activity. Of note, we have established that several immunomodulators exhibit marked efficacy on therapeutic treatment.

a. We recommend that several model infections be used. These should include Caraparu virus, if it is deemed important to evaluate efficacy against a severe hepatic infection that does not involve the CNS. The Banzi and Semliki forest virus infections produced somewhat similar results. However, we believe that Banzi would be better for assessing therapeutic efficacy, since only alpha IFN exhibited partial efficacy against SFV if treatment was delayed until 24 hr after infection.

b. High priority should be given to evaluating combination therapy with immunomodulators and antiviral agents, or with two immunomodulators that have apparently different modes of action.

c. Some effort should be given to developing more sensitive methods for evaluation of antiviral efficacy in vivo, especially those that do not require a lethal endpoint. We recommend evaluation of changes in body temperature, water and food content, and other behavioral changes that can be quantitated using computer monitored cage observation, in order to establish if there is a close correlation with antiviral efficacy as assessed by lethal endpoint. Establishment of such parameters of measurement would mean: (i) lower challenge doses could be used, mimicking the human situation more closely; (ii) a wider variety of virus models may be able to be evaluated, including viruses important in human illness that do not cause lethal disease in adult mice; and (iii) fewer animals may be able to be used, because longitudinal observations would be made on individual mice, and (iv) more potential antiviral agents may be detected than has possible against the very stringent lethal endpoint system.

2. We recommend that treatment with alpha IFN and poly ICLC be considered for evaluation of clinical antiviral efficacy in the appropriate viral infection. These two immunomodulators provided the broadest spectrum activity in therapeutic regimens. We recommend that evaluation of clinical efficacy be performed in conjunction with obtaining data on viral pathogenesis, nonspecific and specific viral immune responses, and any toxicologic manifestations.

Other immunomodulators that had pronounced antiviral activity that might be considered for clinical evaluation include CL246,738, the pyrimidinones, Ampligen, and beta and gamma IFN. However, the broad spectrum therapeutic activity of these compounds has not been established.

3. We recommend that investigation to define the mechanism of action of immunomodulators continue. More information about the interaction of immunomodulators with the immune system network is necessary in order to: (i) establish the best combinations of agents for antiviral chemotherapy; and (ii) define possible deleterious immunotoxicologic effects. We specifically recommend:

a. Discontinue broad screening of the immunomodulating potential of immunomodulators (immunomodulator profiles). At present there is no consistent correlation between any given immunomodulatory activity that can be routinely measured (e.g. circulating levels of IFN, peritoneal Mϕ activation, splenic NK cell activation) and antiviral efficacy.

b. Establish the effects of protective immunomodulator treatment on nonspecific immune responses, virus specific immune responses, and viral pathogenesis -- during the course of infection in treated and untreated animals. This approach is comparable in materials and labor to establishing immunomodulator profiles, but should produce more pertinent information.

c. Continue to use selective cell and mediator depletion as an experimental tool to establish the contributions of particular cells and mediators to antiviral resistance. Such depletions must include independent, overlapping methodologies, because no single method is sufficiently selective. Moreover, because of the complexity involved in depletion methodology, more information needs to be obtained concerning the depletion systems themselves, in order to interpret properly the results.

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Table 1. Immunomodulators

Name	Composition	Source	AVS Number
Microbials			
C. parvum	Killed bacterial vaccine	Burroughs Wellcome	-
TDM	Trehalose dimycolate	Ribi Chem	2155
MPL	Detoxified monophosphoryl lipid A	Ribi Chem	2153
CWS	Cell wall skeleton from Mycobacterium	Ribi Chem	2154
PA-PE	Pyridine extract from C. parvum	Ribi Chem	2157
Synthetics			
CL246,738	3,6-Bis(2-piperidinoethoxy) acridine	Lederle	-
ABPP	2-NH2-5-Br-6-phenyl-4(3H)-pyrimidinone	Upjohn	2776
AIPP	2-NH2-5-I-6-phenyl-4(3H)-pyrimidinone	Upjohn	2777
ABMP	2-NH2-5-Br-6-methyl-4(3H)-pyrimidinone	Upjohn	2778
MVE-2	Maleic anhydride divinyl ether copolymer	Hercules	-
Ampligen	Mismatched poly rI:rC	Johns Hopkins	2149
Ampligen	Mismatched poly rI:rc	HEM	-
GE-132	2-carboxyethylgermanium sesquioxide		3934
HDPP	DL-2,3,5,6,7,8-hexahydro-8,8-Dimethoxy -2-phenylimidazo(1,2,-A)pyridine hydrochloride	-	2880
S-209	Analog of peptidoglycan	Ribi Chem	2158
Poly ICLC	Polyribonucleotide-lysine carboxy-methylcellulose	-	1761
GLA-60	Lipid A subunit analog	Univ. of Kitasato	-
Recombinant DNA derived compounds			
rMuIFN-G	Murine gamma interferon	Genentech	2351
rHuIFN-A A/D	Human alpha A/D interferon	Hoffman LaRoche	-
rMuIFN-B	Murine beta interferon	Toray Industries	-
rHuIL-1 B	Human beta interleukin 1	Smith Kline & French	-
rHuTNF-A	Human tumor necrosis factor alpha	Genentech	-
rHuCSF-M	Human macrophage colony stimulating factor	Cetus	2269
rMuCSF-GM	Murine granulocyte macrophage colony stimulating factor	Behring	-
Antiviral Agents			
Ribavirin	1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide	-	1
New Ribavirin	Ribavirin analog	-	206

Table 2. Virus Strains

Virus Group	Strain	Source of Pool or Seed	Virus Titers		
			In vitro PFU/ml	In vivo i.c. LD_{50} (PFU/LD_{50})	In vivo i.p. LD_{50} (PFU/LD_{50})
Alphaviruses	Semliki Forest virus L10	MBP	6.8×10^7	Not applicable	$10^{6.8}$ (1.1 PFU/LD_{50})
Flaviviruses	West Nile E101	MBP	1.8×10^9	Not being used <u>in vivo</u>	
	Yellow Fever 17D	MBP, one passage	1.6×10^7	3×10^4 ($33 PFU/LD_{50}$)	No symptoms with undiluted
	Banzi	MBP	2.0×10^8	Not applicable	$10^{8.4}$ (0.8 PFU/LD_{50})
Bunyaviruses	Caraparu	MLP	Does not plaque	Not applicable	$10^{2.4}$
	Oriboca	MLP	4×10^6	Not applicable	$10^{1.2}$ ($2.8 \times 10^$ PFU/LD_{50})
Arenaviruse	Pichinde CoAn3739	Vero	4.5×10^8	Not being used <u>in vivo</u>	
Herpesviruses	HSV-2 MS	RKCC	8.0×10^6	Not applicable	ca. $10^{1.0}$ (ca. $1.0 \times 10^$ PFU/LD_{50})
	HSV-1 Kos	Vero	2.0×10^7	Not being used <u>in vivo</u>	

MBP - 10% W/V infant mouse brain pool. MLP - 10% W/V infant mouse liver pool.

Table 3. Effects of rMuGM-CSF on Peritoneal Cells[a]

Exp. Group	Exp #	Dose	Route	PEC/ mouse ($x10^6$) ± SE		Ectoenzyme Specific Activity (in n moles/min/mg protein) ± SE	
				MO	LY	5'N	APD
Naive	1	-	-	1.8 ± 0.2	1.5 ± 0.2	11.1 ± 4.1	15.2 ± 3.6
Vehicle	1	-	i.p.	0.8 ± 0.1	0.9 ± 0.1	7.6 ± 3.4	13.9 ± 2.6
rMuCSF-GM	1	10ug[c]	i.p.	1.4 ± 0.2	2.0 ± 0.2	7.9 ± 1.9	11.6 ± 0.3
rMuCSF-GM	1	10ug[c]	sub.c.	1.7 ± 0.2	1.6 ± 0.2	6.2 ± 2.0	18.9 ± 2.8
Naive	2	-	-	2.9 ± 0.1	1.0 ± 0.2	32.8 ± 6.4	18.4 ± 1.4
Vehicle	2	-	i.p.	2.9 ± 0.2	1.5 ± 0.2	14.4 ± 3.1	17.7 ± 1.6
rMuCSF-GM	2	50ug[d]	i.p.	2.9 ± 0.1	1.1 ± 0.1	21.7 ± 7.2	10.3 ± 0.3*[b]
rMuCSF-GM	2	50ug[d]	sub.c.	2.8 ± 0.2	0.9 ± 0.1	20.1 ± 2.2	22.2 ± 0.3

a B6C3F1 female mice, aged 5-7 weeks, were injected one day before sacrifice with GM-CSF or with the GM CSF vehicle.

b * = Statistically significant ($p < 0.05$) as compared with vehicle control.

c 10ug = 1 X 10^5 U/mg protein.

d 50ug = 5 X 10^5 U/mg protein.

Table 4. Effects of rHuCSF-M on Peritoneal Cells[a]

Exp. Group	Exp.#	Dose	Route	PEC/mouse ($X 10^6$)±SE			Ectoenzyme Specific Activity (in nmoles/min/mg protein)±SE	
				MO	LY	PMN	5'N	APD
Naive	1	-	-	3.1 ± 0.8	0.5 ± 0.2	0	17.2 ± 1.0	22.1 ± 10.8
Vehicle(0.2%BSA/PBS)	1	-	i.p.	3.1 ± 0.8	0.4 ± 0.2	0.06 ± 0.2	12.0 ± 0.3	18.9 ± 3.5
rHuCSF-M	1	$4X10^6$U/ml	i.p.	2.8 ± 0.9	0.5 ± 0.3	0.4 ± 0.2	3.1 ± 1.6*b	12.6 ± 2.8
Naive	2	-	-	3.1 ± 0.4	1.2 ± 0.3	0	22.5 ± 3.0	27.6 ± 6.2
Vehicle(PBS)	2	-	i.p.	3.3 ± 0.7	1.1 ± 0.5	0.08 ± 0.3	16.4 ± 1.9	23.1 ± 1.4
rHuCSF-M	2	$4X10^6$U/ml	i.p.	3.6 ± 0.7	1.5 ± 0.5	0.02 ± 0.03	13.8 ± 1.2	20.5 ± 2.1

[a] B6C3F1 female mice, aged 7-8 weeks, were injected one day before sacrifice with rHuCSF-M or with the vehicle.

*b Statistically significant ($p < 0.05$) as compared with vehicle control.

Table 5. Effect of Immunomodulator Treatment on Intrinsic Resistance of Kupffer Cells to Infection with Pichinde Virus.[a]

Treatment				Mean $\log_{10}$ PFU/ml ± SEM	
Group	Dose	Route	Day	24hr	48hr
Naive	-	-	-	3.03 ± 0.13[*b]	4.14 ± 0.02[*]
C. parvum	35mg/kg	i.p.	-7	5.44 ± 1.00	2.93 ± 0.18[*]
MVE-2	50mg/kg	i.p.	-7	3.71 ± 0.17[*]	2.51 ± 0.17[*]
Control					
Vero Cells (positive control)				6.31 ± 0.49	6.45 ± 2.43

[a] Kupffer cells were isolated from B6C3F1 female mice treated as indicated, and infected the next day with Pichinde virus at a multiplicity of infection of 5.

[b] Statistically significant ($p < 0.05$) from positive control Vero cells.

Table 6. Extrinsic Antiviral Activity of Peritoneal Macrophages Against Pichinde Virus

Treatment of Mice				Mean log_{10} reduction in Pichinde Titer ± SE
Drug	Dose	Route	Day	
Naive	-	-	-	1.0 ± 0.05*
MVE-2	50mg/kg	i.p.	-1	1.39 ± 0.14*
Ampligen	4mg/kg	i.p.	-1	1.48 ± 0.10*
CL246,738	200mg/kg	p.os	-1	1.47 ± 0.14*
ABMP	200mg/kg	i.p.	-1	1.0 ± 0.06*
ABMP	200mg/kg	p.os	-1	0.87 ± 0.03
rHuIFN-A $^{A}/_{D}$	10,000IU (0.14ug)	i.p.	-1	1.4 ± 0.29*
rMuIFN-G	10,000IU (1.34ug)	i.p.	-1	1.67 ± 0.15*

The yields of Pichinde in Vero cells cultured without macrophages ranged from 6.7 to 7.3 log_{10} over four experiments.

* = $p < 0.05$ as compared with Vero cells cultured without Mϕ.

Table 7. Extrinsic Antiviral of Peritoneal macrophages Against Semliki Forest Virus[a]

Mϕ Added	Dose/mouse	Route	Log_{10} PFU ± SE	Log_{10} Difference from control
None			8.1 ± 0.21	
Res	-	-	7.2 ± 0.11	0.9
C. parvum	35mg/kg	i.p.	8.0 ± 0.15	0.1
rHuCSF-M	9.7×10^5U	i.p.	7.9 ± 0.49	0.2
ABPP	200mg/kg	i.p.	6.9 ± 0.03	1.2[b]

[a] BHK cells were infected with Semliki Forest virus, nonadsorbed virus removed with washing, no Mϕ (control) or Mϕ from variously treated mice added, nonattached Mϕ removed with washing, and cultures incubated for 48 hr.

[b] * = $p < 0.01$ as compared with BHK cells cultured without Mϕ.

Table 8. Effect of Immunomodulators on HSV-2(Strain MS) Infection[a]

Drug	Drug Treatment: Dose	Schedule (days)	Route	Vehicle	Mortality: Dead/ Total	Mortality: (%)	Survival: MST	Survival: VR[b]
Synthetic immunomodulators								
Ampligen(T'so)	4mg/kg	0 to +6	i.p.	NaCl	1/10	(10%)*	>21.0*	2.1*
Ampligen(T'so)	4mg/kg	+1 to +6	i.p.	NaCl	0/10	(0%)*	>21.0*	1.8*
Ampligen(T'so)	4mg/kg	+2 to +6	i.p.	NaCl	1/10	(10%)*	>21.0*	2.4*
Ampligen(T'so)	4mg/kg	+2 to +8	i.p.	NaCl	1/10	(10%)*	>21.0*	1.9
Recombinant Derived Biologicals								
rHuIFN-A A/D	5,800IU(0.16ug)	-1	i.p.	PBS/0.2% BSA	5/10	(50%)*	>28.0*	2.1*
rHuIFN-A A/D	5,800IU(0.16ug)	-1 to +6	i.p.	PBS/0.2% BSA	1/10	(10%)*	>28.0*	3.4*
rHuIFN-A A/D	5,800IU(0.16ug)	0 to +2	i.p.	PBS/0.2% BSA	1/10	(10%)*	>28.0*	3.0*
rHuIFN-A A/D	5,800IU(0.16ug)	+1 to +2	i.p.	PBS/0.2% BSA	1/10	(10%)*	>28.0*	2.0*
rHuIFN-A A/D	7,600IU(0.4ug)	+2 to +6	i.p.	PBS/0.2% BSA	1/10	(10%)*	>20.0*	1.7
rMuIFN-B	3,400IU(0.33ug)	-1	i.p.	PBS/0.2% BSA	7/10	(70%)*	9.7*	1.5*
rMuIFN-B	3,400IU(0.33ug)	-1 to +6	i.p.	PBS/0.2% BSA	1/10	(10%)*	>28.0*	3.5*
rMuIFN-B	3,400IU(0.33ug)	0 to +2	i.p.	PBS/0.2% BSA	4/10	(40%)*	>28.0*	2.4*
rMuIFN-B	3,400IU(0.33ug)	+1 to +2	i.p.	PBS/0.2% BSA	1/10	(10%)*	>28.0*	1.7
rMuIFN-B	8,200IU(1.5ug)	+2 to +6	i.p.	PBS/0.2% BSA	0/10	(0%)*	>20.0*	1.9
rMuIFN-G	6,800IU(0.85ug)	-1	i.p.	PBS/0.2% BSA	8/10	(80%)	10.0*	1.4*
rMuIFN-G	6,800IU(0.85ug)	-1 to +6	i.p.	PBS/0.2% BSA	0/10	(0%)	>28.0*	3.5*
rMuIFN-G	6,800IU(0.85ug)	0 to +2	i.p.	PBS/0.2% BSA	5/10	(50%)*	>28.0*	2.0*
rMuIFN-G	6,800IU(0.85ug)	+1 to +2	i.p.	PBS/0.2% BSA	1/10	(10%)*	>28.0*	1.8
rMuIFN-G	800IU(1.70ug)	+2 to +6	i.p.	PBS/0.2% BSA	2/10	(20%)*	>20.0*	1.5

[a] B6C3F1 female mice, aged 6-7 weeks old, were treated as indicated and infected on day 0 i.p. with 2.6×10^4 - 8.33×10^4 PFU (10 LD_{50} doses) of HSV-2. The mortality of the MVE-2 immunomodulator control groups ranged from 0-20% and the MST from >20.0 to >21.0 days. The mortality in the placebo groups ranged from 70-100% and the MST from 7.8 to 10.5 days.

[b] VR = $\frac{\text{Geometric mean time to death of experimental group}}{\text{Geometric mean time to death of placebo group}}$

* = Statistically significant ($p < 0.05$) as compared with the corresponding placebo group.

Table 9. Effect of Immunomodulators on Banzi Flavivirus Infection[a]

Drug	Drug Treatment: Dose	Schedule (days)	Route	Vehicle	Mortality: Dead/ Total	(%)	Survival: MST	VR[b]
Synthetic Immunomodulators								
Ampligen(T'so)	4mg/kg	+1 to +6	i.p.	NaCl	3/10	(30%)*	>19.0*	2.2*
Ampligen(T'so)	4mg/kg	+2 to +8	i.p.	NaCl	8/10	(80%)	12.5*	1.4
Poly ICLC	1mg/kg	+1 to +6	i.p.	NaCl	4/10	(40%)*	>19.0*	2.1*
Poly ICLC	1mg/kg	+2 to +8	i.p.	NaCl	8/10	(80%)	12.5*	1.4
ABMP	200mg/kg	-1	i.p.	1% CMC	10/10	(100%)	8.7	0.9
ABMP	200mg/kg	0	i.p.	1% CMC	10/10	(100%)	8.7	1.1
ABMP	200mg/kg	0 to +2	i.p.	1% CMC	10/10	(100%)	8.6	1.0
ABMP	200mg/kg	-1	p.os	1% CMC	10/10	(100%)	8.5	0.9
ABMP	200mg/kg	0	p.os	1% CMC	10/10	(100%)	8.7	0.9
ABMP	200mg/kg	0 to +2	p.os	1% CMC	10/10	(100%)	8.8	1.1
ABPP	200mg/kg	-1	i.p.	1% CMC	8/10	(80%)	10.0*	1.4
ABPP	200mg/kg	0	i.p.	1% CMC	9/10	(90%)*	11.8*	1.4
ABPP	200mg/kg	0 to +2	i.p.	1% CMC	4/10	(40%)*	>18.0*	1.8
ABPP	200mg/kg	-1	p.os	1% CMC	9/10	(90%)	10.6*	1.3
ABPP	200mg/kg	0	p.os	1% CMC	7/10	(70%)	12.0*	1.5
ABPP	200mg/kg	0 to +2	p.os	1% CMC	7/10	(70%)	14.0*	1.7
Recombinant Derived Biologicals								
rHuIFN-A $^{A}/_{D}$	10,000IU(1.05ug)	0 to +6	i.p.	PBS/0.2% BSA	0/10	(0%)*	>20.0*	2.2*
rHuIFN-A $^{A}/_{D}$	2,200IU(0.40ug)	+1 to +6	i.p.	PBS/0.2% BSA	4/10	(40%)*	>18.0*	1.8*
rHuIFN-A $^{A}/_{D}$	10,000IU(1.05ug)	+1 to +6	i.p.	PBS/0.2% BSA	1/10	(10%)*	>20.0*	2.1*
rHuIFN-A $^{A}/_{D}$	2,200IU(0.40ug)	+2 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	8.7	1.0

Table 9.- continued Effect of Immunomodulators on Banzi Flavivirus Infection[a]

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR[b]
rMuIFN-B	10,000IU(2.45ug)	0 to +6	i.p.	PBS/0.2% BSA	2/10	(20%)*	>20.0*	1.9
rMuIFN-B	10,000IU(2.45ug)	+1 to +6	i.p.	PBS/0.2% BSA	2/10	(20%)*	>20.0*	1.9
rMuIFN-G	10,000IU(5.15ug)	0 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	11.0*	1.3
rMuIFN-G	8,600IU(1.34ug)	+1 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	9.7	1.2
rMuIFN-G	10,000IU(5.15ug)	+1 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	10.7	1.2
rMuIFN-G	8,600IU(1.34ug)	+2 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	8.6	1.0
rHuCSF-M	4.0 X 10^7U/kg	-4 to +2	i.p.	PBS/0.2% BSA	10/10	(100%)	9.7	0.9

[a]B6C3F1 female mice, aged 5-7 weeks old, were treated as indicated and infected on day 0 i.p. with 0.2 to 1.8 X 10^1 PFU (2.5 to 25 LD50 doses) of Banzi virus. The mortality in the MVE-2 immunomodulator control groups ranged from 0-50% and the MST from >17.0 to >20.0. The mortality in the placebo groups ranged from 87-100% and the MST from 8.4 to 9.6 days.

[b] VR = $\frac{\text{Geometric mean time to death of experimental group}}{\text{Geometric mean time to death of placebo group}}$

* = Statistically significant ($p<0.05$) as compared with the corresponding placebo group.

Table 10. Effect of Immunomodulators on Semliki Forest Virus Infection[a]

Drug	Drug Treatment: Dose	Schedule (days)	Route	Vehicle	Mortality: Dead/Total	Mortality: (%)	Survival: MST	Survival: VR[b]
Synthetic Immunomodulators								
Ampligen (T'so)	0.5mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Ampligen (T'so)	2mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Ampligen (T'so)	4mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Ampligen (T'so)	4mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>13.0*	2.3*
Ampligen (T'so)	4mg/kg	+1 to +6	i.p.	NaCl	9/10	(90%)	5.8	1.0
Ampligen (T'so)	4mg/kg	+1 to +6	i.p.	NaCl	8/10	(80%)	6.0*	1.3
Ampligen (T'so)	4mg/kg	+2 to +6	i.p.	NaCl	5/10	(50%)	>13.0*	1.5
Ampligen (T'so)	4mg/kg	+2 to +8	i.p.	NaCl	10/10	(100%)	6.2	1.0
Ampligen (HEM)	0.5mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Ampligen (HEM)	2mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Ampligen (HEM)	4mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Poly ICLC	0.5mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Poly ICLC	1mg/kg	0 to +6	i.p.	NaCl	0/10	(0%)*	>14.0*	4.3*
Poly ICLC	1mg/kg	+1 to +6	i.p.	NaCl	8/10	(80%)	6.3	1.4
GLA-60	0.5mg/kg	-3,0	i.v.	PBS	6/10	(60%)*	7.0*	2.0*
GLA-60	1.5mg/kg	-3,0	i.v.	PBS	3/10	(30%)*	>19.0*	3.2*
GLA-60	0.5mg/kg	-1	i.v.	PBS	6/10	(60%)	6.8	1.3
GLA-60	1.5mg/kg	-1	i.v.	PBS	4/10	(40%)	>19.0	1.9
Recombinant Derived Biologicals								
rHuIFN-A A/D	250IU(0.40ug)	+1 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	6.0	1.0
rHuIFN-A A/D	2,000IU(0.24ug)	+1 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	6.0*	1.1*
rHuIFN-A A/D	10,000IU(2.6ug)	+1 to +6	i.p.	PBS/0.2% BSA	5/10	(50%)	>18.0*	2.4*
rHuIFN-A A/D	250IU(0.40ug)	+2 to +6	i.p.	PSB/0.2% BSA	8/9	(89%)	6.4	1.1
rHuIFN-A A/D	2,000IU(0.24ug)	+2 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	5.8*	0.9
rHuIFN-A A/D	10,000IU(2.6ug)	+2 to +6	i.p.	PBS/0.2% BSA	5/10	(50%)	>18.0*	1.6

Table 10. - continued Effect of Immunomodulators on Semliki Forest Virus Infection[a]

Drug	Drug Treatment Dose	Schedule (days)	Route	Vehicle	Mortality Dead/ Total	(%)	Survival MST	VR[b]
rMuIFN-B	1,870IU(0.33ug)	-1 to +6	i.p.	PBS/0.2% BSA	0/10	(0%)*	>18.0*	2.7*
rMuIFN-B	10,000IU(0.79ug)	0	i.p.	PBS/0.2% BSA	0/10	(0%)*	>18.0*	3.7*
rMuIFN-B	1,870IU(0.33ug)	0 to +6	i.p.	PBS/0.2% BSA	2/10	(20%)*	>18.0*	2.3*
rMuIFN-B	500IU(0.59ug)	+1 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	5.6	1.0
rMuIFN-B	10,000IU(0.79ug)	+1 to +6	i.p.	PBS/0.2% BSA	6/10	(60%)	7.0	1.4
rMuIFN-B	11,600IU(0.33ug)	+1 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	6.0	1.2
rMuIFN-B	500IU(0.59ug)	+2 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	5.5	1.0
rMuIFN-B	11,600IU(0.33ug)	+2 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	5.8	1.1
rMuIFN-G	2,000IU(1.32ug)	-1 to +6	i.p.	PBS/0.2% BSA	0/10	(0%)*	>18.0*	2.7*
rMuIFN-G	2,000IU(1.32ug)	0 to +6	i.p.	PBS/0.2% BSA	1/10	(10%)*	>18.0*	2.5*
rMuIFN-G	2,600IU(1.34ug)	+1 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	6.5	1.2
rMuIFN-G	3,000IU(1.02ug)	+1 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	5.7	1.1
rMuIFN-G	2,600IU(1.34ug)	+2 to +6	i.p.	PBS/0.2% BSA	8/10	(80%)	6.5	1.2
rMuIFN-G	3,000IU(1.02ug)	+2 to +6	i.p.	PBS/0.2% BSA	9/10	(90%)	5.8	1.1
rHuCSF-M	4.0 X 10^7IU/kg	-4 to 0	i.p.	PBS/0.2% BSA	8/10	(80%)	6.6*	1.5
rHuCSF-M	4.0 X 10^7IU/kg	-4 to +2	i.p.	PBS/0.2% BSA	9/10	(90%)	6.6	1.2

[a] B6C3F1 female mice, aged 5-7 weeks old, were treated as indicated and infected on day 0 i.p. with 3.4 to 9.1 PFU (3-10 LD50 doses) of Semliki Forest virus. The mortality in the MVE-2 immunomodulator control groups ranged from 0-50% and the MST from >13.0 to >19.0 days. The mortality in the placebo groups ranged from 80-100% and the MST from 5.4 to 6.8 days.

[b] VR = $\frac{\text{Geometric mean time to death of experimental group}}{\text{Geometric mean time to death of placebo group}}$

* = Statistically significant ($p<0.05$) as compared with the corresponding placebo group.

Table 11. Effect of Immunomodulators on Caraparu Bunyavirus Infection

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR
Synthetic Immunomodulators:								
Ampligen (T'so)	4mg/kg	-1 to +6	i.p.	NaCl	7/10	(70%)	6.7*	1.6
Ampligen (T'so)	4mg/kg	-1 to +6	i.p.	NaCl	8/10	(80%)	8.6*	1.1
Ampligen (T'so)	25mg/kg	-1 to +6	i.p.	NaCl	10/10	(100%)	6.5	1.2
Ampligen (HEM)	4mg/kg	-1 to +6	i.p.	NaCl	5/10	(50%)	14.0*	2.2*
Poly ICLC	1mg/kg	-1 to +6	i.p.	NaCl	1/10	(10%)*	>14.0*	4.3*
Poly ICLC	1mg/kg	-1 to +6	i.p.	NaCl	1/10	(10%)*	>14.0*	4.0*
Recombinant Derived Biologicals:								
rHuIFN-A	1400IU(0.14ug)	-1 to +6	i.p.	PBS/0.2%BSA	9/10	(90%)	6.6*	1.4
rMuIFN-B	186IU(0.79ug)	-1 to +6	i.p.	PBS/0.2%BSA	7/10	(70%)	6.7	1.6
rMuIFN-B	360IU(0.33ug)	-1 to +6	i.p.	PBS/0.2%BSA	10/10	(100%)	6.6*	1.2
rMuIFN-B	5200IU(1.4ug)	-1 to +6	i.p.	PBS/0.2%BSA	9/10	(90%)	6.0	0.6
rMuIFN-B	5200IU(1.4ug)	0 to +6	i.p.	PBS/0.2%BSA	10/10	(100%)	5.6	0.5
rMuIFN-G	540IU(0.49ug)	-1 to +6	i.p.	PBS/0.2%BSA	6/10	(60%)	6.7*	1.8
rMuIFN-G	3000IU(1.02ug)	0 to +6	i.p.	PBS/0.2%BSA	7/10	(70%)	6.5*	1.6
rMuIFN-G	3000IU(1.02ug)	+2 to +6	i.p.	PBS/0.2%BSA	9/10	(90%)	5.7	1.2
rHuCSF-M	4.0 x 10 /kg	-4 to +2	i.p.	PBS/0.2%BSA	6/10	(60%)	6.8	1.4

Table 11.-Continued Effect of Immunomodulators on Caraparu Bunyavirus Infection

Antiviral Agents:								
Ribavirin	100mg/kg	0 to +6	i.p.	PBS	0/10	(0%)*	>19.0*	4.2*
Ribavirin	100mg/kg	0 to +6,8,10, 12,14,16	i.p.	PBS	0/10	(0%)*	>18.0*	4.2*
Ribavirin	100mg/kg	+1 to +6	i.p.	PBS	0/10	(0%)*	>19.0*	3.8*
Ribavirin	100mg/kg	+1 to +6,8,10, 12,14,16	i.p.	PBS	0/10	(0%)*	>18.0*	4.2*
AVS 206	200mg/kg	0 to +6	i.p.	PBS	0/10	(0%)*	>20.0*	5.0*

B6C3F1 female mice, aged 5-6 weeks, were treated as indicated and infected i.p. with 2.5 to 15 LD50 doses of Caraparu virus. The mortality of the MVE-2 immunomodulator control groups ranged from 50%-70% and the MST from 6.5 to >19.0 days. The mortality in the placebo groups ranged from 50-100% and the MST from 4.5 to >14.0 days.

VR=Geometric mean time to death of expermental group

Geometric mean time to death of placebo group

*=Statistically significant ($p<0.05$) as compared to the corresponding placebo group.

Table 12. Effect of Combination Treatment on Caraparu Bunyavirus Infection[a]

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR[b]
Combination Treatments (plus single treatment controls):								
Ampligen	4mg/kg	0 to +6	i.p.	NaCl	9/10	(90%)	6.6	1.1
rMuIFN-G	500IU(0.26ug)	0 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	5.0	0.9
Ribavirin	100mg/kg	+2 to 6,8,10, 12,14,16	i.p.	PBS	17/20	(85%)	6.8	1.2
Ampligen+ rMuIFN-G	4mg/kg+ 500IU(0.26ug)	0 to +6/ 0 to +6	i.p.	NaCl/ PBS/0.2% BSA	8/10	(80%)	6.6	1.2
rMuIFN-G Ribavirin	500IU(0.26ug)+ 100mg/kg	0 to +6 +2 to +6,8,10, 12,14,16/	i.p.	PBS/0.2% BSA PBS	3/10	(30%)*	>20.0*	3.1*
rMuIFN-G	500IU(0.19ug)	0 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	5.2*	1.1
Ribavirin	100mg/kg	+2 to +6,8,10, 12,14,16	i.p.	PBS	10/10	(100%)	5.4*	1.2
rMuIFN-G+ Ribavirin	500IU(0.19ug)+ 100mg/kg	0 + +6/ +2 to +6,8,10, 12,14,16	i.p.	PBS/0.2% BSA/ PBS	4/10	(40%)*	>20.0*	5.0*
rMuIFN-G	500IU(0.19ug)	+2 to +6	i.p.	PBS/0.2% BSA	10/10	(100%)	4.8*	1.1
rMuIFN-G+ Ribavirin	500IU(0.19ug)+ 100mg/kg	+2 to +6/ +2 to +6,8,10, 12,14,16	i.p.	PBS/0.2% BSA PBS	9/9	(100%)	5.6*	1.3
rMuIFN-G	1,000IU(0.39ug)	+2 to +8	i.p.	PBS/0.2% BSA	10/10	(100%)	5.5	1.0
Ribavirin	100mg/kg	+2 to +6,8,10, 12,14,16	i.p.	PBS	10/10	(100%)	5.8	1.0

Table 12. - continued Effect of Combination Treatment on Caraparu Bunyavirus Infection[a]

Drug Treatment					Mortality		Survival	
Drug	Dose	Schedule (days)	Route	Vehicle	Dead/ Total	(%)	MST	VR[b]
rMuIFN-G+ Ribarin	1,000IU(0.39ug)+ 100mg/kg	+2 to +8/ +2 to +6,8,10, 12,14,16	i.p.	PBS0.2% BSA/ PBS	7/10	(70%)	6.3*	1.5
Ribavirin	100mg/kg	+2 to +6,8,10, 12,14,16	i.p.	PBS	7/10	(70%)	6.0	1.3
rMuIFN-G	2,500IU(0.40ug)	+2 to +8	i.p.	PBS/0.2% BSA	8/10	(80%)	5.4	1.0
Poly ICLC	1mg/kg	+2 to +8	i.p.	NaCl	8/10	(80%)	5.0	1.0
Poly ICLC Ribavirin	1mg/kg+ 100mg/kg	+2 to +8/ +2 to +6,8,10, 12,14,16	i.p.	NaCl/ PBS	8/10	(80%)	5.7	1.2
Poly ICLC+ rMuIFN-G	1mg/kg+ 2,500IU(0.40ug)	+2 to +8/ +2 to +8	i.p.	NaCl/ PBS/0.2% BSA	9/10	(90%)	4.6	1.1

[a] B6C3F1 female mice, aged 5-6 weeks, were treated as indicated and infected on day 0 i.p. with 2.5 to 15 LD_{50} doses of Caraparu virus. The mortality of the MVE-2 immunomodulator control groups ranged from 50-70% and the MST was significantly increased in all experiments to 8.0 to >19.0 days. The mortality in the placebo groups ranged from 80-100% and the MST from 4.5 to 6.7 days.

[b] VR = $\frac{\text{Geometric mean time to death of experimental group}}{\text{Geometric mean time to death of placebo group}}$

* = Statistically significant ($p < 0.05$) as compared to the corresponding placebo group.

Table 13. Induction of Interferon by Immunomodulators[a]

			Interferon Titer in plasma (IU/ml)				Interferon Titer in Peritonal fluid (IU/ml)			
Drug	Dose	Route	3hr	6hr	12hr	24hr	3hr	6hr	12hr	24hr
Naive	-	-	<45	<45	<45	<45	<45	<45	<45	<45
MVE-2	50mg/kg	i.p.	<45	<45	<45	<45	<45	<45	<45	<45
Ampligen	4mg/kg	i.p.	135	<45	<45	<45	<45	<45	<45	<45
ABMP	200mg/kg	i.p.	<45	<45	<45	<45	<45	<45	<45	<45
ABMP	200mg/kg	p.os	<45	<45	<45	<45	<45	<45	<45	<45
rHuIFN-$A^A/_D$	10,000IU	i.p.	<45	<45	<45	<45	65	65	<45	<45
Poly ICLC	1mg/kg	i.p.	1100	2900	ND	717	<6	22	ND	17

[a] Female B6CBF1 mice were injected with immunomodulators three hours before the first samples were collected. Interferon was measured by using a standard plaque reduction assay.

Table 14. In Vitro Neutralization of Interferon with Anti-Interferon Serum[a]

Group	Interferon Titer (IU/ml) after addition of: NaCl	NSS	5000IU Anti-IFN	500IU Anti-IFN	50IU Anti-IFN
rHuIFN-A $^{A}/_{D}$	922	346	346	461	364
MuIFN-A,B	173	346	<7	<7	230
Poly ICLC	86	86	<7	<7	43

[a] CD-1 female mice were injected i.p. with 1mg/kg Poly ICLC. After 4 hours plasma samples were collected and a 1:5 dilution of plasma was added to the indicated neutralizing units of anti-interferon, saline, or normal sheep serum. rHuIFN-A and MuIFN A,B titers were adjusted to an estimated 500 IU/ml before addition of anti-interferon, saline, or normal sheep serum. Interferon was measured by using a standard plaque reduction assay after samples were incubated one hour at 4 C.

Table 15. In Vivo Neutralization of Interferon with Anti-Interferon Serum[a]

Drug	Dose	Interferon Titer in Plasma (IU/ml) 1hr	3hr	4hr	20hr
NSS+	-	ND	<6	ND	ND
rHuIFN-A $^{A}/_{D}$	500IU	ND	<6	ND	ND
Anti-IFN+ rHuIFN-A $^{A}/_{D}$	10,000IU+ 500IU				
NSS+	-	ND	ND	50	13
rHuIFN-A $^{A}/_{D}$	13,000IU	ND	ND	<4	<4
Anti-IFN+ rHuIFN-A $^{A}/_{D}$	50,000IU+ 13,000IU				
NSS+	-	ND	ND	7,936	202
Poly ICLC	1mg/kg	ND	ND	7,936	<4
Anti-IFN+ Poly ICLC	50,000IU+ 1mg/kg				
NSS+	-	ND	ND	<4	<4
CL246,738	50mg/kg	ND	ND	<4	<4
Anti-IFN+ CL246,738	50,000IU+ 50mg/kg				
NSS+	-	<4	ND	ND	ND
rHuIFN-A $^{A}/_{D}$	4,000IU	<4	ND	ND	ND
Anti-IFN+ rHuIFN-A $^{A}/_{D}$	100,000IU+ 4,000IU				
NSS+	-	ND	ND	2,434	ND
Poly ICLC	1mg/kg	ND	ND	1,217	ND
Anti-IFN+ Poly ICLC	100,000IU+ 1mg/kg				
NSS+	-	ND	ND	<4	<4
CL246,738	50mg/kg	ND	ND	<4	<4
Anti-IFN+ CL246,738	100,000IU+ 50mg/kg				
NSS+	-	ND	ND	<4	<4
ABMP	200mg/kg	ND	ND	<4	<4
Anti-IFN+ ABMP	100,000IU+ 200mg/kg				
NSS+	-	ND	ND	<4	<4
MVE-2	50mg/kg	ND	ND	<4	<4
Anti-IFN+ MVE-2	100,000IU+ 50mg/kg				

[a] B6C3F1 female mice were injected i.p. with anti-IFN or normal sheep serum (NSS) 4 hours before the i.p. injections of the drugs. Interferon was measured by using a standard plaque reduction assay.

Table 16. Effect of 10,000 Neutralizing Units of Anti-Interferon Serum and Immunomodulators on Semliki Forest Virus Infection[a]

Drug	Drug Treatment: Dose	Vehicle	Mortality: Dead/Total	Mortality: (%)	Survival: MST
Controls:					
Normal Sheep Serum(NSS)	-	NaCl	8/10	(80%)	5.8
Anti-Interferon(Anti-IFN)	10,000IU	NaCl	15/15	(100%)	4.8
Anti-Interferon(Anti-IFN)	2,000IU	NaCl	15/15	(100%)	5.2
Experimental Groups:					
rHuIFN-A $^{A}/_{D}$	40,000IU	PBS/0.2%BSA	0/10	(0%)*	>14.0*
Anti-IFN+ rHuIFN-A $^{A}/_{D}$	10,000IU+ 40,000IU	NaCl/ PBS/0.2%BSA	0/10	(0%)*	>14.0*
Ampligen	4mg/kg	NaCl	1/10	(10%)*	>14.0*
Anti-IFN+ Ampligen	2,000IU+ 4mg/kg	NaCl/ NaCl	0/10	(0%)*	>14.0*
MVE-2	50mg/kg	PBS	0/10	(0%)*	>14.0*
Anti-IFN+ MVE-2	2,000IU+ 50mg/kg	NaCl/ PBS	0/10	(0%)*	>14.0*
Poly ICLC	1mg/kg	NaCl	0/10	(0%)*	>14.0*
Anti-IFN+ Poly ICLC	10,000IU+ 1mg/kg	NaCl/ NaCl	0/10	(0%)*	>14.0*
ABMP	200mg/kg	1% CMC	4/10	(40%)	>14.0
Anti-IFN+ ABMP	2,000IU+ 200mg/kg	NaCl/ 1%CMC	8/9	(89%)	5.1

[a] B6C3F1 female mice, aged 5 weeks, were treated i.p with the indicated dose of anti-IFN serum or NSS 4hr prior to i.p. injection of the immunomodulator, and infected 24hr later with 4.5 PFU (4 LD50 doses) of Semliki Forest Virus.

* = Statistically significant ($p<0.05$) as compared with the corresponding placebo group.

Table 17. Effect of 50,000 Nutralizing Units of Anti-Interferon Serum and Immunomodulators on Semliki Forest Virus Infection

	Drug Treatment		Mortality		Survival
Drug	Dose	Vehicle	Dead/ Total	(%)	MST
Controls:					
Normal Sheep Serum(NSS)	-	NaCl	9/10	(90%)	6.6
Anti-Interferon (Anti-IFN)	50,000IU	NaCl	10/10	(100%)	5.1
Experimental Groups:					
NSS+	-	NaCl/	0/10	(0%)*	>13.0*
rHuIFN-A	13,600IU	PBS/0.2%BSA			
Anti-IFN+	50,000IU+	NaCl/	1/10	(10%)*	>13.0*
rHuIFN-A	13,600IU	PBS/0.2%BSA			
NSS+	-	NaCl/	0/10	(0%)*	>13.0*
Poly ICLC	1mg/kg	NaCl			
Anti-IFN+	50,000IU+	NaCl/	3/10	(30%)*	>13.0*
Poly ICLC	1mg/kg	NaCl			
NSS+	-	NaCl/	0/10	(0%)*	>13.0*
Ampligen	4mg/kg	NaCl			
Anti-IFN+	50,000IU+	NaCl/	5/10	(50%)	6.0*
Ampligen	4mg/kg	NaCl			
NSS+	-	NaCl/	2/10	(20%)*	>13.0*
CL246,738	50mg/kg	H2O			
Anti-IFN+	50,000IU+	NaCl/	3/10	(30%)*	>13.0*
CL246,738	50mg/kg	H2O			
NSS+	-	NaCl/	5/10	(50%)	>13.0
ABMP	200mg/kg	1%CMC			
Anti-IFN+	50,000IU+	NaCl/	6/10	(60%)	6.0
ABMP	200mg/kg	1%CMC			

B6C3F1 female mice, aged 5 weeks, were treated with anti-IFN or NSS 4 hours before i.p. injection of immunomodulators, and infected 24 hours later with 4.5 PFU (5 LD50 doses) of Semliki Forest virus.

*=Statistically significant ($P<0.05$) as compared with the corresponding placebo group.

Table 18. Effect of 100,000 Neutralizing Units of Anti-Interferon Serum and Immunomodulators on Semliki Forest Virus Infection*

Drug	Drug Treatment: Dose	Drug Treatment: Vehicle	Mortality: Dead/Total	Mortality: (%)	Survival: MST
Controls:					
Normal Sheep Serum(NSS)	-	NaCl	8/10	(80%)	5.8
Anti-Interferon (Anti-IFN)	100,000IU	NaCl	8/10	(80%)	4.8
Experimental Groups:					
NSS + rHuIFN-A $^{A}/_{D}$	- 4,000IU	NaCl/ PBS/0.2%BSA	0/10	(0%)*	>19.0*
Anti-IFN+ rHuIFN-A $^{A}/_{D}$	100,000IU+ 4,000IU	NaCl/ PBS/0.2%BSA	0/10	(0%)*	>19.0*
NSS+ Poly ICLC	- 1mg/kg	NaCl/ NaCl	1/6	(17%)*	>19.0*
Anti-IFN+ Poly ICLC	100,000IU+ 1mg/kg	NaCl/ NaCl	2/10	(20%)*	>19.0*
NSS+ Ampligen	- 4mg/kg	NaCl/ NaCl	0/10	(0%)*	>19.0*
Anti-IFN+ Ampligen	100,000IU+ 4mg/kg	NaCl/ NaCl	5/5	(100%)	5.2
NSS+ CL246,738	- 50mg/kg	Nacl/ H_2O	2/10	(20%)*	>19.0*
Anti-IFN+ CL246,738	100,000IU+ 50mg/kg	NaCl/ H_2O	10/10	(100%)	4.6
NSS+ AHMP	- 200mg/kg	NaCl/ 1%CMC	6/10	(60%)	6.0
Anti-IFN+ AHMP	100,000IU+ 200mg/kg	NaCl/ 1%CMC	7/10	(70%)	4.7

Table 18. - continued Effect of 100,000 Neutralizing Units of Anti-Interferon Serum and Immunomodulators on Semiliki Forest Virus Infection[a]

Drug Treatment			Mortality		Survival
Drug	Dose	Vehicle	Dead/ Total	(%)	MST
NSS+ MVE-2	- 50mg/kg	NaCl/ PBS	2/10	(20%)*	>19.0*
Anti-IFN+ MVE-2	100,000IU+ 50mg/kg	NaCl/ PBS	9/10	(90%)	5.5

[a] B6C3F1 female mice, aged 6 weeks, injected i.p. with anti-IFN or NSS 4 hours before the i.p. injection of the immunomodulator and infected i.p. 24 hours later with 4.5 PFU (4 LD50 doses) of Semliki Forest virus.

* = Statistically significant ($p<0.05$) as compared with corresponding placebo group.

Table 19. Intrinsic Antiviral Resistance of Res MØ

Virus	Virus yield /cell at Hours after Infection 2-4	24	72-120
Banzi	0.006	0.004	0.001
Semliki Forest	0.13	0.008	0.0005
Pichinde	ND	0.07	0.01
HSV-1	0.11	0.05	0.01
Yellow Fever	0.001	<0.0002	<0.0002
West Nile	0.007	<0.0002	<0.0002
Oriboca	0.01	0.001	ND
Caraparu	<0.0005	ND	<0.005

16 mm diameter wells containing approximately 2 x 10^5 Res MØ were infected with 2-5 m.o.i. for all viruses except Oriboca and Caraparu. Because of the lower titers of those virus stocks, the estimated m.o.i. was 0.04 for Oriboca and <0.001 for Caraparu virus. At the times indicated cell culture fluid and cells (HSV-1 and Pichinde) or fluid only (the other viruses) were harvested for titration of virus, and the cells were counted. During infection there was no apparent decrease in cell numbers and no visible CPE. All viruses except Oriboca and Caraparu were titrated for PFU. The titers of Oriboca and Caraparu represent suckling mouse LD_{50} doses when inoculated by the i.c. route.

Table 20. Summary of Immunomodulator Profile Data

	Regimen			Splenic NK Cell Activ.	Serum IFN/ Ind.	Peritoneal Macrophage Activation				
	Dose	Route	Schedule			Ectoenzyme Changes		Antitumor Activity	Antiviral Activity[a]	
						5'N	APD		Int.	Ext.
Placebo (Res)	-	-	-	-	-	-	-	-	+	+
MVE-2	50mg/kg	i.p.	D-1	-	-	++	++	++	+	+
C. parvum	35mg/kg	i.p.	D-7	-	-	++	++	++	+	+
CL246,738	100mg/kg	p.os	D-1	++	+	+	+	+	+	+
Ampligen	4mg/kg	i.p.	D-1	++	+	+	+	+	+	+
rMuIFN-G	10,000 IU	i.p.	D-1	++	+	+	+	++	+	+
rHuIFN-A $^{A}/_{D}$	10,000 IU	i.p.	D-1	++	+	+/-	+/-	+/-	+	+

[a] All of the MD groups have shown intrinsic and extrinsic activity against some virus systems.

Table 21. Characteristics of Nonparenchymal, 2 Hour Adherent, and 24 Hour Adherent Cell Populations.

	NPC	2 hour adherent	24 hour adherent
%Mϕ	25	30	65
%LEC	35	65	30
%LY	30	0	0
%Other	10	5	5
% + Fc Recep	ND	ND	95
%Phagocytic	ND	ND	60
% + Ovalb Uptake	ND	70	10
% + C3b Recep.	30	ND	ND

Table 22. Summary of Antiviral Efficacy of Immunomodulators

Immunomodulator	HSV-2 Treatment P &/or P/T	HSV-2 Treatment T	BV Treatment P &/or P/T	BV Treatment T	SFV Treatment P &/or P/T	SFV Treatment T	CV Treatment P &/or P/T	CV Treatment T
Synthetics								
CL246,738	++	++	++	+	++	ND	+	+
ABPP	++	ND	++	++	++	ND	-	ND
AIPP	++	ND	++	ND	++	ND	-	ND
ABMP	-	ND	+/-	-	++	ND	-	ND
MVE-2	++	-	++	ND	++	ND	++	ND
Ampligen	++	++	++	++	++	++	+	-
Poly ICLC	ND	ND	ND	++	ND	++	++	ND
GE-132	-	ND	ND	ND	ND	ND	ND	ND
HDPP	-	ND	ND	ND	ND	ND	ND	ND
S-209	-	ND	ND	ND	ND	ND	ND	ND
GIA-60	ND	ND	ND	ND	++	ND	ND	ND
Recombinants								
rMuIFN-G	++	++	ND	-	++	++	+	+
rHuIFN-A A/D	++	++	ND	++	++	++	+	+/-
rMuIFN-B	++	++	ND	++	++	++	-	-
rHuIL-1 B	-	ND	ND	ND	ND	ND	-	ND
rHuTNF A	-	ND	-	ND	ND	ND	+	ND
rHuCSF-M	-	ND	-	ND	-	ND	-	ND
Microbials								
C. parvum	++	ND	++	ND	++	ND	++	ND
TDM	++	ND	ND	ND	ND	ND	ND	ND
MPL + TDM	++	ND	ND	ND	++	ND	ND	ND
CWS + MPL + TDM	++	ND	ND	ND	++	ND	ND	ND
PA-PE	-	ND	ND	ND	ND	ND	ND	ND
Antivirals								
Ribavirin	ND	ND	++	++	-	-	++	++

Abbreviations: P = single prophylactic treatment; P/T = repeated prophylactic and therapeutic treatment; = repeated therapeutic treatment. ++ = a significant reduction in mortality and a significant increase in median survival time (MST); + = a significant increase in MST only; +/- = inconsistant protection; HSV-2 = herpes simplex virus type 2; BV = Banzi Flavivirus; SFV = Semliki Forest Alphatogavirus; CV = Caraparu Bunyavirus.

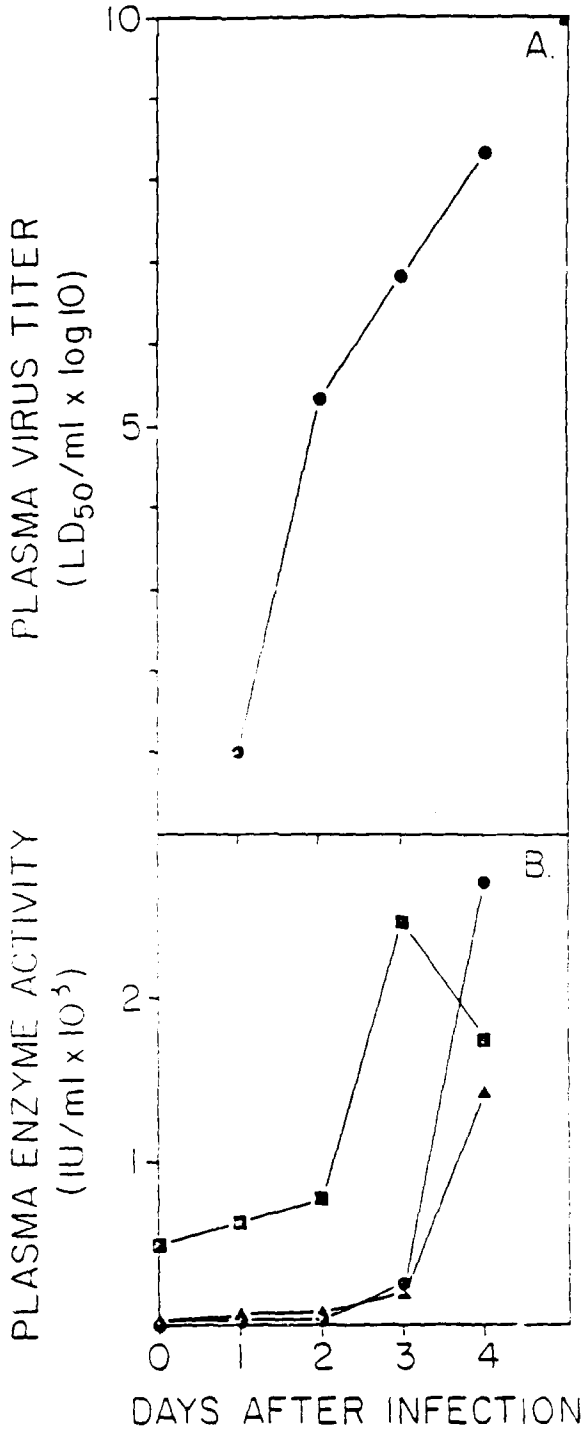

Figure 1. Elevation of virus and enzyme levels in plasma after infection. A. Virus amount (LD_{50}/ml) as determined by titration in 3 day old mice. B. Plasma levels of LDH (■——■), SGPT (●——●), and SGOT (▲——▲).

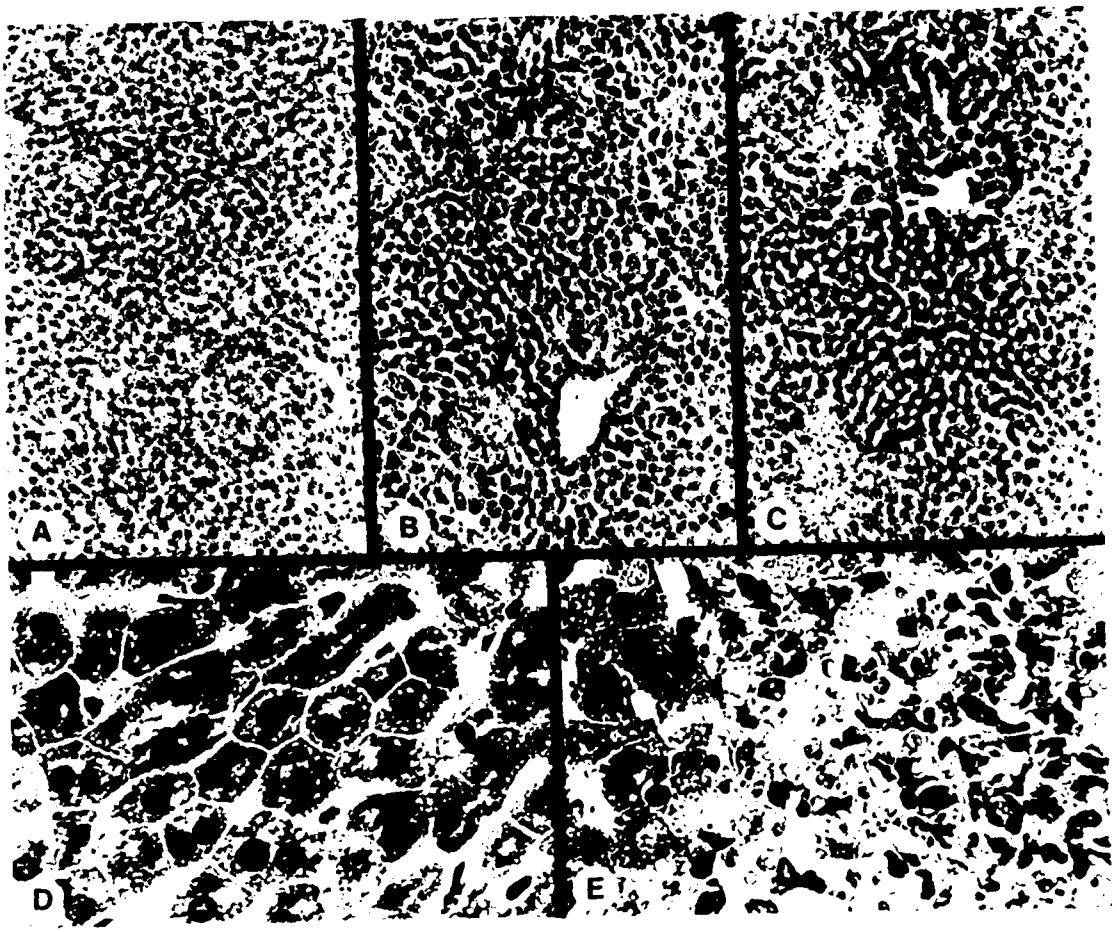

Figure 2. Histopathology of liver tissue. A. and D. On days one and two after infection liver tissue appeared normal. B. On day 3 areas of coagulative necrosis (arrows) were observed in 1/2 to 3/4 of the thin sections examined. C. On day 4 areas of extensive necrosis (arrows) were observed in all thin sections examined. E. Necrosis was characterized by disrupted hepatocyte plasma membranes and nuclei and by coagulation of cytoplasm. Magnification: A to C x 39; D and E x 460.